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(54) Title: TEST OF HIV-SPECIFIC T LYMPHOCYTE FUNCTION THAT DETECTS EXPOSURE TO HIV ANTIGENS AND POSSIBLY EARLY HIV INFECTION

(57) **Abstract**

Methods for the detection of a T cell response in a patient to an antigen from an exogenous source are described. The antigen may be from any non-self source, but the method is particularly advantageous for detection of exposure to agents which do not produce rapid antibody responses. The method is particularly advantageous in detecting exposure to HIV and to other agents where early detection of exposure is important. The method detects activation of T cells in the absence of an antibody response.

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TEST OF HIV-SPECIFIC T LYMPHOCYTE FUNCTION THAT DETECTS EXPOSURE TO HIV ANTIGENS AND POSSIBLY EARLY HIV INFECTION

RELATED APPLICATIONS

This application is a Continuation-In-Part of co-pending Application Serial Number 08/229,108, which in turn is a Continuation of Application Serial Number 07/882,078, now abandoned. Serial Number 07/882,078 was in turn a Continuation-In-Part of Application Serial Number 07/751,998, filed August 29, 1991. Application Serial Number 07/882,078 was also a Continuation-In-Part of U.S. Patent Application Serial Number 07/148,692, filed January 26, 1988. All of these applications are herein incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to a method for the early detection of exposure to infectious organisms. The diagnostic test is based upon the measurement of activation of T cells obtained from a patient by incubation of the T cells with antigenic peptides derived from antigens of such an organism.

In particular, the emphasis of the disclosure is upon application of the method to the detection of exposure of a patient to Human Immunodeficiency Virus (HIV).

Description of the Related Art

Numerous references are made throughout this application to various articles of scientific literature. Such articles are incorporated herein, in their entirety, by such reference.

T lymphocyte immunity may play an important role in the host's response to HIV infection. Natural infection and vaccination against the human immunodeficiency virus type 1 (HIV-1) generally induce both cellular immunity and antibodies (D.E. Lewis et al., Int Rev Immunol 7:1-13 (1990); C.O. Tacket et al., AIDS Res Hum Retroviruses 6:535-542 (1990); S. Abrignani et al., Proc Natl Acad Sci USA 87:6136-6140 (1990); D. Zagury et al., Nature 332:728-731 (1988)). However, the temporal relationship between the appearance of humoral and cellular immunity and their relative roles in protection have not been defined. T cell immunity appears to be transient and declines simultaneously with other immune functions in the HIV-infected host as disease progresses (F. Plata et al., Cytotoxic T lymphocytes in HIV-induced disease: Implications for therapy and vaccination,pp. 401-417 in Immunodeficiencies, F. S Rosen and M. Seligmann eds., c. 1993 by Harwood Academic Publishers, New York; Clerici, M., and G. M. Shearer, *Immunol. Today.*, in press (1994); S.A. Kalams et al., Clinics in Laboratory Medicine 14:271-299 (1994)). In previous studies, we used a series of synthetic amphipathic HIV-1 peptides which are immunogenic for murine (K.B. Cease et al., Proc Natl Acad Sci USA 84:4249-4253 (1987); H. Takahashi et al., J. Exp. Med. 171:571-576 (1990); P.M. Hale et al., Int Immunol 1:409-415 (1989)) and human (J.A. Berzofsky et al., Nature 334:706-708 (1988); M. Clerici et al., Eur J Immunol 21:1345-1349 (1991)) T cells to demonstrate T helper cell (TH) and cytotoxic T lymphocyte responses to HIV-1 in naturally infected HIV-1 seropositive men (M. Clerici et al., Nature 339:383-385 (1989); M. Clerici et al., J.Immunol. 146:2214-2219 (1991)). Using these peptides, we also noted the appearance of a TH response prior to the development of an antibody response to HIV-1 or evidence of virus infection by polymerase chain reaction (PCR) in a high risk individual who later seroconverted (M.

Clerici et al., J Infect Dis 164:178-182 (1991)).

Several lines of evidence suggest that cytotoxic T lymphocyte (CTL) activities might be involved in delaying the onset of disease in HIV-infected patients (A. Hoffenbach et al., J. Immunol. 142:452-462 (1989); F. Buseyne et al., J. Immunol. 150:3569-3581 (1993); A. Carmichael et al., J. Exp. Med. 177:249-256 (1993)). Large numbers of circulating CTL and CTL precursor cells are present in asymptomatic HIV-infected (HIV+) individuals. Circulating CTL numbers decrease gradually with time, such that HIV-specific CTL become undetectable prior to major clinical deterioration (B.D. Walker et al., Nature 328:345-348 (1987); B.D. Walker et al., Science 240:64-66 (1988)). A balance between T cell responses and HIV titers appears to occur during latent infection and AIDS. Evidence for CD8+ cytotoxic T lymphocyte precursors specific for cells expressing HIV-1 *gag*, *pol* and *env* with clearance of viremia in acute infection suggests that cellular immunity is involved in the initial control of virus replication in primary HIV infection and imply a role for CTL in protective immunity to HIV *in vivo* (R.A. Koup et al., J. Virol. 68:4650-4655 (1994); J.T. Safrit et al., J. Exp. Med. 179:463-472 (1994)). Further evidence for a role of CTL activity in the delay of disease progression has been provided in studies on animal models of SIV and HIV infection (G. Voss et al., J. Acq. Immunodef. Synd. 6:969-976 (1993); N.L. Letvin et al., Semin. Immunol. 5:215-223 (1993)).

Several lines of evidence suggest that exposure to HIV does not necessarily result in seroconversion or infection defined by standard criteria. Nevertheless, several laboratories have recently reported that exposure to HIV, in the absence of seroconversion, can induce HIV-specific cell mediated immune responses that have been suggested to contribute to virus clearance (A.M. Ranki et al., AIDS 3:83-89 (1989); W. Borkowsky et

al., AIDS Res. Hum. Retrovir. 6:673-678 (1990); M. Clerici et al., J. Infect. Dis. 164:178-182 (1991); M. Clerici et al., J. Infect. Dis. 165:1012-1019 (1992); R. Cheynier et al., Eur. J. Immunol. 22:2211-2217 (1992); H.C. Keller et al., AIDS Res. Hum. Retrovir. 8:1355-1359 (1992); S.L. Rowland-Jones et al., Lancet 341:860-861 (1993); A. DeMaria et al., J. Infect. Dis. 170:1296-1299 (1994); M. Clerici et al., AIDS 7:1427-1433 (1993); M. Clerici et al., JAMA 271:42-46 (1994); P. Langlade-Demoyen et al., J. Clin. Invest. 93:1293-1297 (1994)). Accumulating evidence suggests that a rapid and effective CTL response during an invasive exposure to HIV might be involved in clearing the organism of the first infected cells. Unusually high frequencies of HIV-specific CTL precursors have been demonstrated also in uninfected donors, presumably related to priming by cross-reactive antigens such as homologous bacterial and viral proteins, HLA antigens and other self proteins as well as endogenous retroviral proteins (A. Hoffenbach et al., J. Immunol. 142:452-462 (1989). It is plausible that rapid recruitment and expansion of these cells after limited HIV exposure could account for the protection against HIV infection. In this context, strong HIV specific CTL activity has been recently reported in uninfected infants born from HIV-infected mothers (R. Cheynier et al., Eur. J. Immunol. 22:2211-2217 (1992); S.L. Rowland-Jones et al., Lancet 341:860-861 (1993); A. DeMaria et al., J. Infect. Dis. 170:1296-1299 (1994)). Furthermore, high frequency of CTL precursors against *nef* antigen was recently demonstrated in uninfected individuals sexually exposed to HIV (P. Langlade-Demoyen et al., J. Clin. Invest. 93:1293-1297 (1994)).

SUMMARY OF THE INVENTION

The invention comprises a diagnostic test wherein the response of TH cells obtained from a patient to

peptides derived from antigens of an infectious agent is assessed. Though the application refers to experiments performed for assay of HIV exposure, the methods disclosed can be applied to any virus or other agent for which immunogenic peptides can be identified.

In the experiments described herein, production of interleukin-2 (IL-2) by T cells in response to HIV-1 peptides implies specific sensitization to envelope glycoprotein (env) epitopes as a result of immunization or infection. However, other indications of TH cell activation can be assessed, for instance T cell proliferation or production of cytokines other than IL-2.

In other embodiments of the invention, the activation of populations of T cells, other than T helper cells is assessed. For example, the killing activity of cytotoxic T lymphocytes (CTL) may be measured. In yet additional embodiments, both CTL killing activity and T helper activity are measured.

Also, production of any cytokine, rather than interleukin-2 might be measured as a means of assessing immune response. In yet other embodiments of the invention, cytokine determination might be performed by immunoassay rather than by measuring proliferation of a cell line.

The invention provides a method of detection of exposure to HIV that is an alternative to the measurement of antibody titer to HIV antigens (seroconversion) or PCR amplification of viral DNA. The test described by the present invention can be used to assess exposure to HIV without concomitant persistent infection. As such, it is a valuable tool in any survey of HIV infection, as it may be used to identify individuals who, by virtue of mounting a prompt cellular immune response (perhaps to a low multiplicity infection), are able to defeat infection by HIV. Such individuals would not be identified in the tests

currently in use. For instance, the PCR amplification of viral DNA requires persisent infection to detect exposure. Furthermore, the test provides an earlier identification of HIV exposure than is provided by seroconversion, as T helper response precedes antibody production in the chain of events leading to the humoral immune response. The test may be particularly useful in the screening of the blood supply, as it detects exposure prior to seroconversion.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 show interleukin-2 (IL-2) production from lymphocytes from (A) a typical human immunodeficiency virus type I (HIV-1) seropositive donor and (B) a typical HIV-1 seronegative heterosexual control.

Figure 2A-2E shows interleukin-2 (IL-2) production from the five recently exposed seronegative men (A-E) at the first (top), second (two months later, middle) and third visits (six months after the first visit, bottom). Proliferation of the CTLL is shown as counts per minute (cpm) using supernatant dilutions of 1:2, 1:4, 1:8 and 1:16 from cultures of peripheral blood monocytes (PBMC) and each peptide or medium without peptide.

Figures 3A to 3H shows CTL activity of cultures stimulated with env peptides against autologous EBV-transformed targets pulsed with the stimulating peptide (⊟,⊖) or with RPMI (O, ▵). Donor 1-7 (panel A-G) had been exposed to body fluids from HIV-infected patients. The donor shown in Fig. 3H is representative of donors exposed to body fluids from a seronegative patient. (A) Donor 1: CTL response of cultures stimulated with P18 IIIB 01,O) or with a pool of T1/T2 peptides (O, ▵). (B) Donor 2: CTL response of cultures stimulated with a pool of T1/T2 peptides. (C) Donor 3: CTL response of cultures stimulated with a pool of P18 MN/M peptides. (D) Donor 4: CTL response of cultures stimulated with the P18 MN peptide. (E) Donor

5: CTL responses of cultures stimulated with a pool of T1/T2/Th4.1 peptides. (F) Donor 6: CTL responses of cultures stimulated with the T I peptide. (G) Donor 7: CTL responses of cultures stimulated with a pool of T1/T2/Th4.1 peptides. (H) CTL responses from cultures stimulated with T1/T2/th4.1 or P18 MN/IIIB pools of peptides. Donor 1: HLA-A2,33, B27,60, C-, DR2, 4, w53, DQ 1,3. Donor 2: HLA-A 2,28, B18, B50, Cw6, w7, DR7, 11, w52,w53 DQw2,7. Donor 3: BLA-A28, 33, B4, B57, Cw6,7. Donor 4: HLA-Al, A-, B7,62, Cw3,w7, DR1,4, w53 DQ5,8. Donor 5: HLA-Al, 11, B44, 55, Cw3, DR1,6,w52, DQ1. Donor 6: HLA-A10,24, B18,-, Cw5, DR11, 15, w52, DQw6, w7. Donor 7: HLA-A2,33;B7,38; C-; DR11,15,w52, DQ1,3.

Figure 4A shows the CTL activity of a T1/T2 peptide pool-stimulated culture assayed against autologous (▥) (Donor 2: HLA-A2,28; B18,50; Cw6,w7; DR7,11,w52, w53, DQw2,7) and HLA-mismatched EBV targets (▨); (Donor 5: HLA-A1,11; B44,55; Cw3,w5, DR1,6,w52; DQ1) peptide-pulsed targets.

Figure 4B shows the CTL activity of a P18 MN-stimulated culture assayed against autologous (Donor 4) media-pulsed targets (▧) and peptide-pulsed targets in the presence (□) or absence (▨) of anti-class I W6/32 mAb (25 μg/ml). The W6/32 monoclonal antibody did not inhibit T cell proliferation to tetanus toxoid.

Figure 5A to 5B shows the specificity of peptide-induced CTL responses. 5A; CTL activity of T1 (▤), T2 (▨), Th4.1 (▩), P18MN (▧) or P18 IIIB (□)-stimulated cultures against targets pulsed with a pool of T1,T2,Th4.1,P18MN and P18IIIB at an effector:target ratio of 60:1. 5B; CTL activity of T1/T2/Th4.1 (▧) or P18MN/IIIB (▦)-stimulated cultures against T1/T2/Th4.1 or P18MN/IIIB-stimulated targets respectively, at an effector:target ratio of 60:1.

Figures 6A to 6E show the relationship between env-specific CTL activity (o) and T helper responses (▲) in

CTL responders at different time points after exposure to HIV. 6A; Donor 1: CTL activity of a TI/T2-stimulated culture against T1/T2-pulsed autologous EBV targets and T1/T2-induced IL-2 response. 6B; Donor 1: CTL activity of a P18 IIIB-stimulated culture against P18 IIIB-pulsed EBV targets and P18 IIIB-induced IL-2 response. 6C; Donor 2: CTL activity of a T1/T2-stimulated culture against T1/T2-pulsed targets and T1/T2-induced IL-2 rsponse. 6D; Donor 3: CTL activity of a P18 MN/IIIB peptide pool-stimulated culture against P18 MN/IIIB-pulsed EBV targets and P18 MN/IIIB-pulsed IL-2 response. 6E; Donor 5: CTL activity of a T1-stimulated culture against T1-pulsed targets and T1-induced IL-2 response.

Figures 7A to 7C show T1 and P18 MN-specific CTL activity of CD8+ cell lines generated from CD8+ cells isolated from the PBMC of Donor 6, 425 days after HIV exposure. The CTL were generated during four weekly rounds of stimulation with T1 or P18MN. Lysis of autologous EBV targets pulsed with: T1 (●), Th4.1 (▲) or media (□) (6A); or P18 MN (●), P18 IIIB (▲) or media (□) (6B). 5C shows a comparison of lysis of autologous EBV targets pulsed with P18 MN (●) or media (■) with that of HLA-mismatched EBV-transformed targets pulsed with P18 MN (▲) or media (□).

DETAILED DESCRIPTION OF THE INVENTION

The Examples below set forth specific studies conducted to determine exposure of a patient to particular infectious agents. Tests based upon immune responses to peptides derived from the envelope glycoprotein of HIV-1 are particularly emphasized. However, it is important to understand that the general concept of the tests can be applied to diagnosing exposure to any infectious agent, provided that proper antigenic peptides can be identified. The means for identifying peptides that represent antigens from an infectious agent are broadly described in Example 1.

The peptides used in experiments described in the working examples were identified essentially as described in Example 1, using HIV-1 as the pathogen of interest. The peptides can be used individually, but can also be used as pools of peptides. Use of pooled peptides is preferred in assessment of CTL responses.

Once one or more antigenic epitopes are identified for an infectious agent, then these epitopes can be synthetically produced and utilized in the method of the invention to determine patient exposure to that infectious agent. In particular, the identified antigen is substituted for the HIV-1 envelope peptides in the methods set forth in the Examples below. Incubation of PBMC from the patient with the antigen and measurement of activation of the PBMC as a result of the incubation provides a method of diagnosing patient exposure to the infectious agent.

The immune response in a patient begins with the residence of a foreign substance, for the purposes herein an infectious agent, inside of "antigen presenting" cells of the host. These cells process the infectious agent and present fragments of its constituents on their cell surface, associated with major histocompatibility receptor molecules (MHC-antigen complexes). These MHC-antigen complexes are in turn recognized by T lymphocytes, which are activated by the recognition event and respond in a number of ways, including cytokine secretion, proliferation, and specific interactions with other immune cells to start the processes of clonal expansion of antibody producing cells. A population of activated T lymphocytes remains in a patient exposed to the antigenic substance which is capable of recognizing that substance in vitro. Thus, activation of these cells can be measured following presentation to these T lymphocytes of the antigen. Such measurement is the basis of the methods described in the Examples below.

This activation process can be utilized according to the present invention to provide early detection of exposure to an infectious agent and is particularly useful in detection of such exposure prior to antibody development. Many diseases, for instance Lyme disease must be diagnosed early in their course, before the time necessary for the antibody response to occur, if effective treatment is to be easily provided.

Example 1: Selection of peptides that induce in vitro T-cell responses in mice of multiple MHC types and in a population of seropositive humans.

This is accomplished for HIV as previously described in U.S. Patents 5,081,226, issued January 14, 1992 and 5,030,449, issued July 9, 1991. Additional information concerning the method for selecting epitopes recognized by T cells can be found in M. Clerici et al., Nature 339:383-385 (1989), J.A. Berzofsky et al., Nature 334:706-708 (1988), K.B. Cease et al., PNAS USA 84:4249-4253 (1987) and P.M. Hale et al., Int. Immunol. 1:409-415 (1989). Some peptides that might be useful in the stimulation of a T cell response to HIV have been described in J.A. Berzofsky et al., J. Clin. Invest. 88:876-884 (1991).

Selection of useful peptides for malarial antigen stimulation of a T cell response has been described in U.S. Patent 4,886,782, issued Dec. 12, 1989 to M.F. Good et al. and also in M.F. Good et al. Science 235:1059-1062 (1987).

A general review of the considerations in identifying appropriate epitopes can be found in Milich, D.R., Adv. in Immunol. 45:195-282 (1989) and also in Cornette, J.L., Methods in Enzymology 178:611-634 (1989).

Example 2: Selection of the experimental population

High risk homosexual men were recruited from the 600 HIV-1 seronegative participants in the UCLA Multicenter AIDS Cohort Study (MACS, ref. (R.A. Kaslow et al., Am J Epidemiol 126:310-318 (1987); J.S. Chmiel et al., Am J Epidemiol 126:568-577 (1987))). Five men with recent exposure to HIV-1 answered an advertisement requesting HIV-1 seronegative MACS participants who had engaged in unprotected receptive anal intercourse twice or more in the last 9 months with an HIV-1 seropositive partner to volunteer for an immunologic study. Data from 20 months of intensive investigation on these men are reported here. The sexual partners of these five patients were not available due to confidentiality restrictions in the MACS. Except for their recent high risk behavior, these men were representative of the MACS cohort in general and did not have a history of other infections or unique behavior which set them apart from the rest. The recruiting process to identify men with admitted recent unprotected anal receptive intercourse with HIV-1 infected partners was necessary because most individuals in the MACS cohort now use condoms with HIV-1 infected and unknown serostatus sexual partners and therefore are unlikely to be recently exposed to HIV-1 (R. Detels et al., J Acquir Immune Defic Syndr 2:77-83 (1989)). Positive controls were 11 asymptomatic HIV-1 seropositive MACS participants. Negative controls were 13 HIV-1 seronegative age-matched heterosexual men from Los Angeles, as well as a larger cohort of 136 low risk seronegative individuals from the Washington, D.C. area. A group of 13 seronegative MACS participants who are referred to here as "moderate" risk were also studied. These men had not (to their knowledge) been exposed to HIV-1 recently through receptive anal intercourse although they had practiced high risk behavior prior to joining the MACS in 1984, and still claim to

practice safer homosexual activities (R. Detels et al., J Acquir Immune Defic Syndr 2:77-83 (1989)).

Example 3: Assessment of TH cell activation in response to HIV-1 derived peptides

Peripheral blood is collected in heparin and mononuclear cells (PBMC) were separated on Ficoll-hypaque. Samples from most of the individuals from Los Angeles were coded and tested blind in Bethesda, in order that no bias could affect the results. Cultures to induce proliferation and IL-2 production were established in triplicate and contain 3 x 10^5 mononuclear cells in 0.2 ml of RPMI + 5% human serum.(M. Clerici et al., Nature 339:383-385 (1989); C.S. Via et al., J Immunol 144:2524-2528 (1990)) Synthetic peptides corresponding to the env protein of HIV-1, shown in Table 1 (SEQ. ID. NO. 1 - 7), which have been described previously (U.S. Patent 5,081,226, issued January 14, 1992 and 5,030,449, issued July 9, 1991, U.S. Patent Application Ser. No. 07/148,692 and K.B. Cease et al., Proc Natl Acad Sci USA 84:4249-4253 (1987); H. Takahashi et al., J. Exp. Med. 171:571-576 (1990); P.M. Hale et al., Int Immunol 1:409-415 (1989); H. Takahashi et al., Proc Natl Acad Sci USA 85:3105-3109 (1988)) were used at 2.5 μM.

Table 1: Antigens, human immunodeficienty virus type 1(HIV-1) and control peptides used in the interleukin-2 (IL-2) generation assays.

	PEPTIDE SEQUENCES	DESCRIPTION
POSITIVE CONTROL FOR T CELL RESPONSE		
Influenza		Influenza A/Bangkok RX73 (H3N2) [15]
HIV-1 GP 160 PEPTIDES		
T1	KQIINMWQEVGKAMYA	HIV-1/III_B Env amino acid residues 428-443 [5,31]
T2	HEDIISLWDQSLK	HIV-1/III_B Env amino acid residues 112-124 [5,31]
TH4.1	DRVIEVVQGAYRAIR	HIV-1/III_B Env amino acid residues 834-848 [7,31]
P18-IIIb	RIQRGPGRAFVTIGK	HIV-1/III_B Env amino acid residues 315-329 [6,16,31]
P18-MN	RIHIGPGRAFYTTKN	HIV-1 (MN) Env region homologous to p18-III_B [32]
NEGATIVE CONTROL PEPTIDES		
P23	KQSSGGDPEIVTHSF	non-immunogenic HIV-1 Env peptide 369-383 [7]
myoglobin	NKALELFRKDIAAKY	immunogenic non-HIV peptide, sperm whale myoglobin amino acid residues 132-146 [33]

Influenza virus (Influenza A/Bangkok RX73 [H3N2] (FLU) prepared as described previously (G.M. Shearer et al., J Clin Invest 74:496-506 (1984)), was used at a maximum stimulatory concentration as a positive control for each individual's CD4 response to a recall antigen. Cultures to assess IL-2 production include 2 mg/ml of anti-IL-2 receptor monoclonal antibody (anti-Tac, ref. (T. Uchiyama et al., J Immunol 126:1393-1397 (1981))), generously provided by Dr. T. Waldmann (Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD) in RPMI 1640 medium (GIBCO) supplemented with 100 U/ml of penicllin G, 100 μg/ml streptomycin, 2 mM L-glutamine and 2% human AB serum (Sigma). Anti-Tac was added at the initiation of culture to retard consumption of IL-2. Cultures to assess proliferation were pulsed at day 6 with 1 mCi of [^{3}H] thymidine and harvested 18 hours later. For proliferation, a stimulation index of greater than two was scored as positive (M. Clerici et al., Nature 339:383-385 (1989)). To assess IL-2 production, culture supernatants are harvested after 7 days, and total IL-2 produced throughout the culture period is determined by testing each supernatant for ability to stimulate the proliferation of an IL-2-dependent mouse continuous T lymphocyte line designated CTLL. Four successive two-fold dilutions in triplicate are set up to test the supernatants for ability to stimulate the proliferation of 8 x 10^3 CTLL/well in 96-well microtiter plates (M. Clerici et al., Nature 339:383-385 (1989); C.S. Via et al., J Immunol 144:2524-2528 (1990); S. Gillis S et al., J Immunol 120:2027-2032 (1978)) in medium as described above with the additional supplement of 5 x 10^{-5} M β-mercaptoethanol and using 10% fetal calf serum in place of the 2% human AB serum. After 24 hr, the CTLL cultures are pulsed with 1 mCi of [^{3}H] thymidine, and harvested 18 hr later. Results are expressed as the mean counts per minute (CPM) for triplicate

wells/supernatant dilution and the entire titration curves are plotted for unstimulated, FLU-stimulated and peptide-stimulated cultures. A response was scored as positive when, at two or more successive supernatant dilutions, the CPM from the peptide-stimulated cultures exceeds the CPM from the unstimulated cultures by a factor of greater than two-fold. At each visit, serologic status of the patients was determined by ELISA (Genetic Systems, Seattle, WA) with confirmation by Western blot against HIV antigens (Novapath Immunoblot Assay, Biorad, Hercules, CA) (P. Nishanian et al., J Clin Microbiol 25:395-400 (1987)), and $CD4^+$ cell number was determined by flow cytometry (J.V. Giorgi et al., Clin Immunol Immunopathol 55:173-186 (1990)). Typing for HLA-A, B, DR and DQ was performed using standard procedures and typing kits (One Lambda, Canoga Park, CA).

IL-2 is produced in response to HIV-1 peptides by PBMC of HIV-1 seropositive men. Weak or absent proliferative response to recall antigens is characteristic of approximately of 50% HIV-1 seropositive individuals (M. Clerici et al., Nature 339:383-385 (1989); J.V. Giorgi et al., J Immunol 138:3725-3730 (1987); G.M. Shearer et al., J Immunol 137:2514-2521 (1986); H.C. Lane et al., N Engl J Med 313:79-84 (1985)). In previous reports, a sensitive assay for IL-2 production was used to examine T cell responses to HIV-1 in naturally infected HIV-1 seropositive (M. Clerici et al., Nature 339:383-385 (1989); M. Clerici et al., J Clin Invest 84:1892-1899 (1989)) or recombinant gp160 vaccinated (M. Clerici et al., Eur J Immunol 21:1345-1349 (1991)) individuals. In the current study, PBMC from HIV-1 seropositive men were tested for ability to proliferate and produce IL-2 in response to the synthetic amphipathic HIV-1 peptides T1, T2, Th4.1 P18 IIIB and P18 MN (see Table 1). An example of IL-2 production by PBMC of an HIV-1 seropositive

individual in response to FLU and to HIV-1 env peptides is shown in Figure 1A. The absence of IL-2 production to HIV-1 peptides from PBMC of a representative low risk, HIV-1 seronegative individual is shown in Figure 1B. These results verify that IL-2 production to HIV-1 peptides can be used to detect specific sensitization to HIV-1. Negative control peptides are P23, a non-immunogenic HIV-1 envelope peptide (P.M. Hale et al., Int Immunol 1:409-415 (1989); H. Takahashi et al., Proc Natl Acad Sci USA 85:3105-3109 (1988)), and an immunogenic myoglobin peptide (I. Berkower et al., J Immunol 136:2498-2503 (1986)).

IL-2 is produced in response to HIV-1 peptides by PBMC of recently exposed high risk seronegative men. The pattern of IL-2 produced in response to the synthetic HIV-1 peptides is shown in Figure 2 for repetitive testing of each of the five high risk seronegative men with recent exposure to HIV-1. At their first test, PBMC from four of the men generated IL-2 in response to the peptides. PBMC from the fifth individual (donor E), generated IL-2 in response to the peptides when he was tested two months later (second test). Four of the five men (subjects A, C, D and E) have remained HIV-1 seronegative (by ELISA and Western Blot) for at least ten months after they were first studied. T cells from three of these men (donors A, C, and D) generated IL-2 in response to at least one of the synthetic HIV-1 env peptides each of the three times they were tested, whereas donor E was positive one of the two times he was tested. In contrast to the IL-2 response, PBMC from none of these five individuals responded to the peptides by proliferation. None of the men had evidence of numerical or functional CD4 deficiency. CD4 counts for these individuals (A, C, D and E) were all within the normal range for our laboratory (mean $\pm$ 1 SD, 1017 $\pm$ 329 CD4/mm^3) (J.V. Giorgi et al., Clin Immunol Immunopathol 55:173-186 (1990)) and

all the PBMC samples (including those designated B(1), B(2) and E) produced IL-2 in response to FLU. None of the lymphocytes tested responded to the negative control peptides, P23 or myoglobin (132-146) (see Table 1).

One of the five individuals (donor B) seroconverted between his first and second visits. He had evidence of a TH response to the HIV-1 env peptides in the absence of an antibody response at his first visit (Fig. 2B(1)), in that his PBMC produced IL-2 in response to four of the HIV-1 peptides. However, at his second visit (Fig. 2B(2)), two months later, he no longer exhibited a TH response to the HIV-1 envelope peptides, although he retained a positive (but reduced) response to FLU. His CD4 counts dropped from 652/mm^3 (Fig. 2B(1)) to 357/mm^3 (Fig. 2B(2)). At his third visit (Fig. 2B(3)), the CD4 count was 332/mm^3, antibody responses on Western Blot were stronger, the FLU-specific TH response remained positive (although reduced), but the HIV-1 envelope T cell response was still absent. A positive IL-2 response to FLU at both the second and the third visits indicated that loss of response to HIV-1 had occurred, but not a generalized loss of the IL-2 responseness to a recall antigen (M. Clerici et al., Nature 339:383-385 (1989); M. Clerici et al., J Clin Invest 84:1892-1899 (1989)).

The results of our experiments including those from eleven seropositive and 149 seronegative control individuals are summarized in Table 2. Lymphocytes from eight of the 11 seropositive men (eight of nine who were responsive to FLU) tested as positive controls produced IL-2 when stimulated with at least one of the HIV-1 envelope peptides. Two of the three HIV-1 seropositive men who did not respond to any of the HIV-1 peptides also did not respond to FLU, suggesting that the self-restricted, CD4-mediated TH pathway required for in vitro production of IL-2 was no longer intact in these men (M. Clerici et al., Nature 339:383-385 (1989); M.

Table 2: Human immunodeficiency virus type 1 (HIV-1) seronegative homosexuals with known rece exposure to HIV-1 displayed specific T-cell immunity in an IL-2 generation assay against synthetic HIV envelope peptides.

TEST GROUPS	POSITIVE CONTROL ANTIGEN:	HIV-1 PEPTIDES:						NEGATIVE CONTR ANTIGENS:	
	Influenza virus	T1	T2	Th4.1	P18IIIB	P18MN	One or more	P23	Myo.*
Recently Exposed Seronegative Homosexuals:	5/5	5/5	4/5	4/5	5/5	4/5	5/5	0/5	0/4
HIV-1 Seropositive Homosexuals:	9/11	5/11	7/11	6/11	7/10	5/10	8/11	N.D.	N.D.
HIV-1 Seronegative Heterosexuals: (Los Angeles cohort)	13/13	3/13	1/13	3/13	2/13	1/13	3/11	0/7	0/1
HIV-1 Seronegative Heterosexuals: (Washington D.C. cohort)	136/136	3/136	0/136	3/136	2/136	1/136	7/136	N.D.	N.D.

Myo., myoglobin
N.D., not determined

Clerici et al., J Clin Invest 84:1892-1899 (1989)). PBMC from none of the seropositive men made substantial proliferative response to the HIV peptides.

Of the 13 heterosexual seronegative controls, three (23%) were positive to one or more peptides. Of these three donors, two responded to two peptides and one responded to four peptides. One of the three donors had been previously exposed to HIV-1 by an accidental needlestick but has remained seronegative for more than two years. A second of these donors, was originally negative to all the peptides but tested positive to four of the peptides two months later. Neither of two other low risk heterosexuals contols who were repetitively tested for peptide-specific TH activity four or more times showed reactivity to any of the petides in any of the tests. Of the 136 heterosexuals seronegative controls studied in the Washington D.C. area, seven (5.1%) individuals were responsive to one or more of the env synthetic peptides. Of these seven individuals, five responded to only one peptide, one responded to two peptides, and one responded to three peptides, whereas the high risk individuals all responded to multiple peptides. It should also be noted that none of the donors tested responded to non-immunogenic HIV env P23 peptide nor to the sperm whale myoglobin peptide (see Table 2). Using a Fisher exact test, the difference between the anti-HIV-1 response frequency of the high risk homosexuals (5 of 5 responded) and the heterosexual controls from the Los Angeles cohort (excluding the known exposed control, 2 of 12 responded) was significant ($p = 0.049$). These differences were even more significant when compared with 7/136 control donors from the Washington, D.C. cohort ($p < 5x10^{-5}$).

HIV-1 T cell immune response was also observed in HIV-1 seronegative homosexual men who did not report recent exposure. Our group of five homosexual men were highly selected and biased toward recent very high risk

behavior. To address the question of how frequent an HIV-1 specific peptide response may be among other MACS participants, a group of 13 randomly selected "moderate" risk seronegative homosexual men who are participants in the Multicenter AIDS Cohort Study were studied. When first tested, two individuals exhibited evidence of T cell sensitization. One of these men was re-tested approximately one year later and retained his response. When eight of the original group of moderate risk men who initially did not produce IL-2 in response to the peptides were re-tested, approximately one year later, five produced IL-2 when stimulated with the peptides. Thus, a cell mediated response to HIV-1 appeared in five of five recently exposed homosexual men, and at least once each in seven of thirteen moderate risk HIV seronegative homosexual men without known recent exposure to HIV.

There is no correlation between HLA type and peptide-induced IL-2 production. We HLA typed PBMC for A, B, DR and DQ from : a) the five recently exposed seronegative homosexuals men; b) the seven moderate risk seronegative homosexual men who responded to the peptides; c) five of the moderate risk seronegative homosexual men who did not respond to the peptides; and d) the seronegative control who had been accidentally exposed to HIV-1 and responded to the peptides. We did not detect any correlation between a particular HLA allele and IL-2 production in response to any of the env peptides (Table 3). For each peptide, there were donors who did not share any DR or DQ molecule, but who all responded to that peptide. These results suggest that each of the peptides can be presented by more than one DR or DQ molecule. However, we cannot exclude the alternate possibility that they are all presented by DP molecules, for which we could not type.

Table 3. HLA typing of scronegative individuals who responded or did not respond to HIV-1 env synthetic peptides.

Donor Identification	HLA antigens expressed by individual: HLA-A	HLA-B	HLA-DR	HLA-DQ	IL-2 response to peptide: T1	T2	Th4.1	PI8IIIB	P18MN
		High risk recently exposed homosexual men							
A	2,-	8,27	1,w17	w5,w2	+	+	+	+	+
B	1,24	8,w75	7,w8	w2,w4	+	+	+	+	+
C	1,2	w62,w57	1,7	w5,w2	+	+	+	+	+
D	24,29	51,-	4,w11	w7,-	+	+	-	+	-
E	11,30	13,35	7,-	w2,-	+	-	+	-	+
		Moderate risk homosexual men who responded to the peptides							
1	1,2	18,22	w11,w13	w6,w7	+	-	+	+	+
2	1,2	51,37	4,w10	w5,w7	+	+	+	+	-
3	2,3	7,w47	w17,4	w3,-	-	-	+	-	-
4	2,3	16,35	w17,w14	w3,w2	-	-	+	-	-
5	24,26	44,w75	w8,w14	w3,w4	-	+	-	+	+
6	2,11	51,8	w13,w12	w6,w7	-	+	-	+	+
7	2,26	27,w60	1,w8	w5,w4	+	+	+	+	-
		Moderate risk homosexual men who did not respond to peptides							
8	2,w68	8,14	w17,7	w2,-	-	-	-	-	-
9	2,32	w63,7	w15,7	w6,w2	-	-	-	-	-
10	2,24	8,45	w17,7	w2,-	-	-	-	-	-
11	2,24	51,50	4,7	w2,w7	-	-	-	-	-
12	1,w68	8,44	17,w11	w2,w7	-	-	-	-	-
		HIV-1 exposed heterosexual man who responded to peptides							
Control	2,30	w50,w60	w14,w17	w2,w5	+	-	+	+	-

Example 4: Assay for HIV infection by polymerase chain reaction

In order to provide independent assessment of the infection status of the experimental subjects of Example 1, quantitative polymerase chain reaction (PCR) analysis for HIV-1 genomic sequences was performed on DNA from 10^5 PBMC as previously described (S. Pang et al.,Nature 343:85-89 (1990); J.A. Zack et al., Cell 61:213-222 (1990)). The oligonucleotide primer pair used is specific for a region of the HIV-1 *nef* gene which overlaps the U3 region of the 3' long terminal repeat (LTR). The sense oligonucleotide primer used was AA943 (5'-TGACTTACAAGGCAGCTATAGATC-3') (SEQ. ID. NO. 8), which corresponds to nucleotides 9048-9061 of the HIV-1 JRCSF isolate (Y. Koyanagi et al., Science 236:819-822 (1987)). The antisense primer was AA946 (5'-CTCTGGATCAACTGGTACTAGC-3') (SEQ. ID. NO. 9) which corresponds to nucleotides 9265-9244. Non-quantitative PCR was performed using LTR AA55/M667 and gag SK38/39 primers as described elsewhere (J.A. Zack et al., Cell 61:213-222 (1990); S.M. Wolinsky et al., Ann Intern Med 111:961-972 (1989)). To determine if the DNA present in the sample was capable of amplification, an aliquot of each coded sample was analyzed for conserved sequence of the β-globin gene for quantitative PCR and of HLA gene for non-quantitative PCR. All PCR tests were performed without knowledge by the laboratories running the tests of the serologic or TH status of each subject.

PCR tests did not detect virus in recently exposed men. To address the question of whether the high risk individuals harbor the HIV-1 genome, we performed quantitative polymerase chain reaction (PCR) analysis (S. Pang et al.,Nature 343:85-89 (1990); J.A. Zack et al., Cell 61:213-222 (1990)) for HIV-1 genomic sequences on donors A, C and D. In one experiment we tested blood samples collected at the time of the third visit (6 months after these men were first T cell responsive to

HIV-1), using an oligonucleotide primer pair specific for a region of the HIV-1 nef gene. We quantitatively detected from 2 to 250 copies of HIV-1 in our positive controls. Whereas DNA from 10^5 cells from two seropositive individuals resulted in signals equivalent to 5 and 25 copies of HIV, the same number of cells from the three recently exposed seronegative men and five additional seronegative controls were negative when assayed in parallel by PCR (data not shown).

In another experiment, PCR was performed using gag SK38/39 and LTR AA55/M667 primers on samples collected at 10 months after the initial visit of donors A, C, D and E. Also tested in this experiment were three specimens from donor B (one collected 3 months prior to visit B, a second collected at the first seropositive visit, B', and a third collected six months after visit B'). As expected, all samples of PBMC in this experiment showed characteristic amplified product for HLA-DQ gene indicating that these samples had amplifiable DNA. No HIV-1 viral DNA was detected in donors A, C , D or E . Also no viral DNA was detected in the pre-seroconversion sample from donor B, or in samples from six other seronegative heterosexual controls, even when 4 mg of input DNA (i.e., 6 x 10^5 cells) was used in the amplification reaction. Positive signals were present with both primer pairs in the PCR test of both post-seroconversion specimens from donor B and in the samples from two seropositive controls. Attempts to isolate HIV-1 from four of these five individuals (A,B,D,E) by co-culture techniques were unsuccessful.

The immune systems of the five recently exposed homosexual men whom we studied had been exposed to sufficient quantities of HIV-1 to induce an anamnestic response in the T cell compartment. IL-2 production in response to these env peptides has now been tested in 149 low risk HIV seronegative individuals (see Table 2)

and spurious cross-reactions of T cells with selected HIV-1 protein epitopes are rare (J.A. Berzofsky et al., Nature 334:706-708 (1988); M. Clerici et al., Nature 339:383-385 (1989); M. Clerici et al., J Clin Invest 84:1892-1899 (1989)). Further evidence that the epitopes recognized were HIV-1 specific was the failure of the PBMC from the men who we tested to generate IL-2 in response to the negative control P23 and myoglobin peptides. This latter observation precludes the possibility that the PBMC from the recently exposed HIV-1 seronegative individuals can be non-specifically activated to generate IL-2 in culture in response to irrelevant antigenic stimulation. The specificity of the IL-2 production in response to these HIV-1 env peptides is supported by an extensive survey of the literature that did not uncover any statistically significant similarities between the HIV-1 peptides used in our study and sequences in the PIR and Swiss-Prot protein libraries (Search performed by Dr. Gerald Myers, Los Alamos National Laboratory).

Although TH from the high risk, recently exposed seronegative men produced IL-2 in response to the HIV-1 peptides, their lymphocytes did not proliferate (incorporate [^{3}H] thymidine) in response to the peptides. This difference in response may be due to the greater sensitivity of the IL-2 production bioassay compared with the proliferation assay, which we have reported previously for this test in asymptomatic seropositive individuals (M. Clerici et al., Nature 339:383-385 (1989)). It is also possible that exposure to HIV-1 antigens under conditions which do not induce antibody production (possibly low dose antigen exposure) would initiate IL-2 production without necessarily inducing T cell proliferation. Finally, it is possible that cytokines produced during HIV infection or alterations in antigen-presenting cell function affect proliferation more than IL-2 production.

In summary, we sought evidence of HIV-specific TH cell responses in the absence of antibody responses in HIV-1 seronegative homosexual men whose sexual behavior placed them at high risk for infection with HIV-1. All five high risk men we studied who had recent sexual exposure to HIV-1 exhibited evidence of *in vitro* TH cell immune activity in response to HIV-1. In addition, peripheral blood mononuclear cells from seven of thirteen homosexual men who were considered to be at moderate risk, exhibited TH cell activity to HIV-1 synthetic peptides. No evidence of virus by culture was found in a group of persistently seronegative, recently exposed high risk men, in whom we detected evidence of T cell sensitization to HIV. Our results differ from those of Imagawa et al. (D.T. Imagawa et al., N Engl J Med 320:1458-1462 (1989)), who isolated virus from high risk seronegative homosexual men using 10^7 cells in a co-culture assay. Virus isolations attempted in the laboratory of Dr. Imagawa (UCLA) on the first visit of four of the high risk men (donors A,B,D,E) were all negative. We also tested two persistently seronegative high risk men from our experimental group for *in vitro* production of anti-HIV antibody using the method of Jehuda-Cohen et al. (T. Jehuda-Cohen et al., Proc Natl Acad Sci USA 87:3972-3976 (1990)), who reported finding circulating B cells that produced antibodies reactive with HIV-1 in a number of seronegative high risk men. Both of the high risk men we tested were negative in this assay, whereas the HIV-1 seropositive controls we tested were strongly positive. Of our study group, one individual became seropositive during the course of the study (see Example 3). Thus, with the exception of one seroconverter, the men we studied have remained seronegative with no evidence of virus infection or B cell responsiveness despite evidence of T cell sensitization.

The IL-2 production assay used in this study

detects T cells with specificity for HIV-1 envelope peptides. This response appears to indicate that an individual has been exposed to HIV-1 or HIV-1 antigen at a level sufficient to induce a T cell response. However, because these men do not have classical markers of HIV-1 infection including anti-HIV-1 antibody, PCR evidence of HIV-1 genome, infectious virus demonstrable by culture, or CD4 immunodeficiency, these men cannot be said to be infected despite evidence of exposure. It is possible that virus may be present in lymph nodes, although not detectable in the peripheral blood. A recent paper (G. Pantaleo et al., Proc Natl Acad Sci USA 88:9838-9842 (1991)) indicating that the level of virus in the lymph nodes can be orders of magnitude higher than the level circulating in the peripheral blood would support this hypothesis.

In the individuals studied, the most intriguing finding is the presence of a cell mediated immune response in the absence of an antibody response. This finding is reminiscent of the experimental observations in animal models of delayed type hypersensitivity in the absence of an antibody response when very low doses of antigen were administered (C.R. Parish et al., J Exp Med 135:298-311 (1972)). It is of interest that Abrignani et al. (S. Abrignani et al., Proc Natl Acad Sci USA 87:6136-6140 (1990)) noted that in the vaccine trial of a nonglycosylated denatured form of HIV-1 gp120 (Env2-3), the lower (50 mg) dose of Env 2-3 was more effective than the higher (250 mg) dose in inducing a cell mediated immune response. Furthermore, we have reported that low dose immunization (40-80 mg) of seronegative volunteers with a rgp160 candidate AIDS vaccine resulted in strong TH responses to the same env peptides used in this study, but no or only transient antibody responses (M. Clerici et al., Eur J Immunol 21:1345-1349 (1991)). In contrast, immunization with higher doses of rgp160 resulted in both cell-mediated

and antibody responses (R. Dolin R, et al., Ann. Int. Med. 114:119-127 (1991) ; E.L. Cooney et al., Lancet i:567-572 (1991)). The observation of a cell mediated response in the absence of a humoral response is compatible with the hypothesis that these men have been exposed to low levels of viral antigens. Alternatively, the presence of T cell sensitization in the absence of an antibody response may in part reflect timing. In vivo, T helper cell responses precede antibody responses, and it is possible that the remaining four seronegative individuals will eventually seroconvert. However, if they do seroconvert, it will not be possible to determine whether seroconversion was the result of the exposure(s) that we detected with peptide-specific TH function or of subsequent HIV infection, since they continue high-risk behavior.

An early immune response to HIV-1 is the most likely explanation for the positive anti-HIV-1 response in the seroconverter in this study, and in the naturally infected participant in a longitudinal study of immune responses to HIV-1 that we reported earlier (M. Clerici et al., J Infect Dis 164:178-182 (1991)). The four recently exposed men in this study who remained seronegative, and possibly the seronegative moderate risk men who were TH responsive to HIV-1 peptides might not be infected. It is possible that they were exposed to HIV-1 or HIV-1 antigens at a level sufficient to prime T cell immunity, but insufficient to induce antibody production. The experiments set forth in the examples provide evidence that specific T cell sensitization to HIV-1 can be repeatedly detected in some exposed individuals in the absence of other positive tests for HIV-1 exposure (antibody) or infection (PCR and viral culture).

Example 6: Longitudinal Study of a Moderate Risk Individual

A 40 year old, homosexual man in good general health was followed longitudinally for a 19 month period. When interviewed intially he indicated that the had been promiscuous in the past, but he had been involved in a monogamous sexual relationship with a seronegative parner for the previous 8 months. He denied history of blood transfusion, intrvenous drug abuse, or sexually transmitted diseases. Blood samples were taken from him at seven intervals during the next 19 months. Serum samples were tested for the presence of antibodies to HIV and p24 antigen and were initially negative; PBMC cultured for HIV at the beginning of the observation period were also negative. PBMC were cryopreserved for later *in vitro* TH cell assays. Throughout the sutdy period, the subject remained healthy and his $CD4^+$ lymphocyte count ranged from 803 to 2586 cells/mm^3.

Blood samples were taken at 0, 1, 5, 10, 13, 16 and 19 months. Serum was frozen at -20°C and assayed for the presence of HIV antibodies by ELISA using a commercial kit (Abbott Laboratories, North Chicago, IL), for p24 antigen by ELISA also using a commercial kit (Abbott), for antibodies to baculovirus-derived recombinant glycoprotein 160 (rgp160, MicroGeneSys, West Haven, CT) by ELISA, and for antibodies to HIV by Western blot using a commercial kit (Du Pont, Doraville, GA). PBMC from months 0, 5, 10, 13, 16 and 19 were cryopreserved in liquid nitrogen. Samples of these frozen cells were thawed at the end of the 19 month period and tested for T cell proliferation. Samples from months 1, 5, 13, 16 and 19 were tested for interleukin-2 production induced by HIV synthetic peptides derived from gp160 (See Table 1 of Example 3). PBMC from months 0, 5, 10, 13, 16 and 19 months were tested for the presence of HIV *gag* sequences by PCR.

The anti-gp160 antibody assay was performed using rgp160 at 0.1 μg/well or control baculovirus-derived protein, also at 0.1 μg/well as the solid phase, as previously described (C.O. Tacket, et al., AIDS Res. Hum. Retroviruses 87:3972 (1990)). This assay has been used to detect the transient, low-titer antibody seen in individuals vaccinated with rgp160.

PCR was performed on PBMC obtained at six different time points using the *gag* primers SK38/39 by C. Yih Ou, (Division of HIV/AIDS, Center for Infectious Diseases, Centers for Disease Control, Atlanta (C.Y. Ou et al., Science 239:295 (1988)).

PBMC from 5 time points were tested for their ability to respond to synthetic peptides of the HIV envelope gp160, as previously reported (M. Clerici et al., Nature 339:383 (1989)). PBMC from months 13, 16 and 19 were teested on samples of fresh blood at the time the samples were taken. Also, cyropreserved PBMC from months 1, 5, 13, 16 and 19 were thawed and tested together in the same experiment. Finally, PBMC taken from rgp160-vaccinated volunteers (M. Clerici et al., Eur. J. Immunol., 21:1345 (1991)) before and after immunization were tested with the peptides as positive and negative controls, respectively. The HIV synthetic peptides used are shown in Table 1 of Example 3.

The ability of PBMC to produce antigen-induced IL-2 or to proliferate was determined by culturing the PBMC at 37°C in a moist, 7% CO_2 atmosphere. PBMC were either unstimulated or stimulated with FLU (1:500 concentration) or HIV synthetic peptides (2.5 μmol/ml final concentration). The culture medium was RPMI1640 (GIBCO, Grand Island, NY). For IL-2 production, 3 x 10^5 PBMC were cultured in 96-well, flat-bottom culture plates (Costar, Cambridge, MA) for 7 days in the presence of 4 μg/ml human anti-IL-2 receptor antibody (anti-Tac) to prevent IL-2 consumption by the stimulated cells. Supernatants were frozen and stored at -20°C

until assayed for IL-2 content. For IL-2 assay, 8×10^3 cells of the IL-2 dependent cell line CTLL were cultured in each well of a 96-well flat-bottom microtiter plate, in the presence of five twofold dilutions of unstimulated or antigen-stimulated culture supernatants, as previously described (M. Clerici et al., Nature 339:383 (1989); M. Clerici et al., J. Clin. Invest. 83:1430 (1989)). After 24 h, the cultures were pulsed with 1 μCi of [^{3}H]-thymidine and harvested after 18 additional hours with a 96-well cell harvester (TOMTEC, Orange, CT). ^{3}H incorporation was determined using an LKB β-plate spectrometer (Pharmacia LKB Biotechnology, Piscataway, NJ). For proliferation studies, 3×10^5 PBMC were cultured for 6 days in 96-well, flat-bottom plates, pulsed for 18 hours with [^{3}H]d-thymidine, and harvested as outlined above.

The results of this longitudinal study are summarized in Table 4 below:

TABLE 4

Comparison of different assays for detection of human immunodeficiency virus type 1 (HIV-1) exposure or infection during an 18-month period before seroconversion.

	Month of test						
	0	1	5	10	13	16	19
T helper cell studies							
IL-2 production to							
T1	NT	-	+	NT	+	+	+
T2	NT	-	+	NT	+	+	+
T4	NT	-	-	NT	+	+	+
P18	NT	-	-	NT	+	+	+
Proliferation to							
T1	NT	-	+	NT	+	NT	+
T2	NT	-	-	NT	+	NT	+
Th4	NT	-	+	NT	+	NT	+
P18	NT	-	+	NT	+	NT	+
Serology							
HIV-1 ELISA	-	-	-	-	-	-	+
rgp 160 ELISA	-	-	-	-	-	-	+
p24 antigen	-	-	-	-	-	-	-
Western blot	-	-	-	-	-	-	+
Polymerase chain reaction	-	NT	-	-	-	-	+

NOTE: Interleukin-2 (IL-2); HIV synthetic peptides *env*T1, *env*T2, *env*Th4, and *env*P18 (T1, T2, Th4, and P18, respectively); not tested (NT); negative (-); positive (+); recombinant gp 160 (rgp 160).

Thus, for >1 year before seroconversion and PCR positivity, cryopreserved PBMC from this subject became responsive to HIV envelope antigenic determinants by two TH cell assays. Standard HIV antibody and PCR tests did not identify HIV infection during the period in which both TH cell assays indicated that he had been exposed to HIV envelope determinants. PCR remained negative during the period of observation, until seroconversion to HIV antigens occurred by month 19.

Example 5: Assessment of CTL killing activity in response to HIV-1-derived peptides

We recently reported T helper cell reactivity to HIV envelope peptides in six out of eight health care workers (HCW) exposed to body fluids from HIV$^+$ patients (M. Clerici et al., JAMA 271:42-46 (1994)), as well as in PCR negative homosexual men who engage in high-risk sexual behavior (M. Clerici et al., J. Infect. Dis. 165:1012-1019 (1992)). However, T helper cell responses could be caused by exposure to defective or dead virus, whereas CD8$^+$ CTL would be strongly suggestive that virus actually infected some cells to be presented with class I MHC molecules. HCW accidentally exposed to HIV represent a unique population for whom the time and type of exposure are specifically recorded. Furthermore, in contrast to other cohorts of HIV-exposed individuals, exposure of HCW should be lower and involve a single event. Although the risk of transmission of HIV in this population is estimated to be low (less than 0.3%) (D.K. Henderson et al., Ann. Intern. Med. 113:740-746 (1990)), it has been demonstrated that HIV can be readily cultured from the infected residual blood aspirated from needles (H. A. Kessler, unpublished observations), suggesting that most high risk percutaneous exposures of HCW can result in live HIV inoculation. To address whether a single documented high risk exposure to HIV results in activation of CTL immunity, we analyzed CTL

activity specific for synthetic peptides corresponding to the envelope (*env*) of HIV in a well characterized group of HCW occupationally exposed to body fluids from HIV^+ patients, in parallel with the evaluation of HIV-specific T helper cell reactivity.

Subjects

Health care workers who received accidental parenteral exposures to body fluids from HIV-infected or uninfected patients reported immediately to the Employee Health Service, Rush Presbyterian-St. Luke's Medical Center, where they were interviewed, completed questionaires concerning their accidents. At that time they were informed of this study and asked whether they wanted to participate. Those who were interested were given a copy of the protocol and consent form. Twenty-eight HCW reporting occupational exposures to HIV-infected blood or body fluids were prospectively enrolled from 9/90 to 5/94. Thirty-eight HCW reporting blood or body fluid exposures from HIV-1 seronegative source patients, as well as 33 healthy blood donors were enrolled as controls (Table 5). The participants were recruited under protocols reviewed and approved by the Institutional Review Boards of both Rush-Presbyterian-St. Luke's Medical Center and the National Cancer Institute (NCI). Whole blood was obtained at various time intervals (range 0-99 weeks) following the occupational exposure. Information about HCW post-exposure management and source patient clinical status was obtained by reviewing HCW employee health records, source patient medical records and the Infectious Disease clinical charts of HCW who elected to take AZT following their injuries, as well as those of the respective source patients.

Synthetic peptides

The peptides used in this study were synthesized as

previously described (K.B. Cease et al., Proc. Natl. Acad. Sci. USA. 84:4249-4253 (1987); H.J. Takahashi et al., Proc Natl Acad Sci. USA. 85:3105-3109 (1988); P.M. Hale et al., Immunol. 1:409-415 (1989)). The peptides, based on the sequence of gp160 HIV-1 IIIB are: *env* T1 (KQIINMWQEVGKAMYA, aa residues 428-443; gp120); *env* T2 (HEDIISLWDQSLK, aa residues 112-124; gp120); *env* Th4.1 (DRVIEVVQGAYRAIR, aa residues 834-848; gp160) and *env* P18 (P18 IIIB; RIQRGPGRAFVTIGK; aa residues 315-329; gp160). An *env* peptide based on the sequence of gp160 HIV-1 MN was also used (P18 MN, RIHIGPGRAFYTTKN; homologous to P18 IIIB; gp160). Peptides were dissolved in RPMI 1640 and stored at -80°C.

Preparation of Mononuclear Cells

Blood was collected into heparinized vacuum tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ) in Chicago, coded and shipped overnight at ambient temperature to the NCI in Bethesda, where the samples were tested. The peripheral blood mononuclear cells (PBMC) were separated by centrifugation at 1800g for 20 minutes on Ficoll-Hypaque gradients. The PBMCs were washed twice in PBS (Gibco) and resuspended in RPMI 1640 supplemented with 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and HEPES buffer (Gibco). In most cases, the samples were coded and tested in a blinded manner such that the investigators in Bethesda did not know which samples came from unexposed individuals or from HCW exposed to HIV^+ or HIV^- body fluids. In most cases, repeat bleeds of the same health care worker carried different code numbers, and the investigators in Bethesda were unable to distinguish between the experimental and control groups.

In vitro assay for T helper function

The production of IL-2 was tested by stimulation of $3x10^5$ PBMC/well (in triplicate) in 96-well microtiter

plates (Costar, Cambridge, MA) in 0.2 ml of RPMI + 5% human serum (M. Clerici et al., Nature 339:383-385 (1989)). Synthetic peptides corresponding to the *env* of HIV-1 (K.B. Cease et al., Proc. Natl. Acad. Sci. USA. 84:4249-4253 (1987); H.J. Takahashi et al., Proc Natl Acad Sci. USA. 85:3105-3109 (1988); P.M. Hale et al., Immunol. 1:409-415 (1989)) were used as previously described at 2.5 μM (M. Clerici et al., Nature 339:383-385 (1989); M. Clerici et al., J. Clin. Invest. 84:1892-1899 (1989)). Influenza A/Bangkok RX73 [H3N2] (FLU) prepared and used at an optimal stimulatory concentration as a positive control for each individual's CD4-mediated response to a recall antigen (28, 29). The cultures also contained 2 μg/ml of the anti-Tac anti-IL-2 receptor monoclonal antibody (generously provided by Dr. John Hakimi, Hoffman-La Roche, Nutley, NJ) to block IL-2 consumption. Culture supernatants were harvested after 7 days. The total IL-2 produced throughout the culture period was determined by testing each supernatant for ability to stimulate proliferation of an IL-2-dependent mouse continuous T lymphocyte line (CTLL). Four successive two-fold dilutions in triplicate were used to test the supernatants for ability to stimulate the proliferation of 8 x 10^3 CTLL/well in 96-well microtiter plates (M. Clerici et al., Nature 339:383-385 (1989)). After 24 hr, the CTLL cultures were pulsed with 1 μCi of [^{3}H] thymidine, and harvested 18 hr later. A sample was scored as positive if there was a positive IL-2 response to two or more of the 5 HIV peptides tested. An IL-2 response was considered positive to a given peptide when the proliferation (in cpm) of CTLL cells in the presence of the supernatants of cultures with the peptides exceeded 5 fold or more the proliferation of cells cultured in the presence of unstimulated cultures.

Results were expressed as the mean counts per minute (CPM) for triplicate wells at the highest

supernatant dilution tested, as well as stimulation index (S.I.) calculated as: cpm of cultures in the presence of peptide-stimulated supernatants minus cpm of cultures in the presence of unstimulated supernatants.

Cytotoxic T lymphocyte assays

Preparation of effector CTL

CTL assays were performed according to the method previously reported (M. Clerici et al., J. Immunol. 146:2214-2219 (1991)). $3x10^6$ freshly isolated PBMC were incubated for 7 days with the HIV *env* synthetic peptides (2.5 μM) at 37° C in a humidified 5% CO2 incubator in RPMI 1640 supplemented with 5% human serum. The cells were then washed and resuspended in RPMI 1640-10% FCS and used in the CTL assays at a concentration of $3x10^6$ cells /ml .

Preparation of target cells

Target cells were EBV-transformed B lymphoblastoid cell lines. Autologous B lymphoblastoid cell lines were generated by incubating PBMCs with the supernatant of B95.8 cells, a cell line that chronically produces Epstein Barr virus, and an anti-CD3 monoclonal antibody (OKT3, Ortho Biotech, Raritan, NJ), provided by the Division of Cancer Treatment, National Cancer Institute. Target cells were labeled with Chromium-51 (150 μCi $Na_2{}^{51}CrO4$, Amersham Corp, Arlington Heights, IL) and pulsed overnight with either no peptide or 5 μM of the peptides. After three washes with PBS, the targets were ressuspended at $5x10^4$ cells/ml in RPMI 1640 containing 10% FCS and were dispensed into the wells of a 96 well round bottom microtiter plate at $5x10^3$cells/well. One hundred μl of effector cells at concentrations of $3x10^5$, $15x10^4$ and $7.5x10^4$ was added in triplicate to the target cells. Spontaneous release was determined in targets cultured in media alone. Maximal release was

determined from 100 μl of each target incubated with 5% Triton X-100. After 6h incubation, supernatants were harvested and counted in a gamma counter (Micromedic Systems Inc., Horsham, PA). Percent specific lysis was determined as 100% x (test cpm-spontaneous cpm)/ (maximum cpm-spontaneous cpm). A CTL response was considered positive when the difference between the % lysis of peptide pulsed targets and % lysis of RPMI-pulsed targets was greater than 15%. In cases of high % lysis against RPMI-pulsed targets, responses were considered positive when the % specific lysis against peptide-pulsed targets was three fold above the RPMI-pulsed targets. Antibody-blocking experiments were performed by incubating target cells with an anti-class I antibody W6/32 (anti-HLA-A,-B,-C) at a fixed E:T.

Generation of HIV-specific $CD8^+$ cell lines

$CD8^+$ cells were isolated using Dynabeads (Dynal Inc., Lake Success, NY) according manufacturer's instructions. $1x10^6$ cells were cultured in the presence of irradiated peptide-pulsed autologous PBMC ($1.5x10^6$ cells/well) in 48 well plates in media supplemented with 10% human serum. Interleukin-2 (10 U/ml, Boehringer Mannheim, Indianapolis, IN) was added 3 days after culture. The cells were restimulated weekly with irradiated autologous peptide-pulsed PBMC and maintained in IL-2 (10U/ml) containing media, that was changed at three-day intervals. CTL assays were performed as described above after four rounds of stimulation.

HIV-1 provirus detection by PCR

PCR as performed according to the manufacturer's instructions (Roche Molecular Systems). Briefly, an ethylenediaminetetracetic acid-anticoagulated blood sample (0.5ml) was processed with specimen wash reagent to lyse the blood red blood cells. The cell pellet was extracted with proteinase K and non-ionic detergents and

amplified with SK431/462 primers (A. Butcher and J. Spadoro, Clin. Immunol. Newslett. 12:73-76 (1992)). To prevent carry over in this system uracil-N-glycosylase was utilized in the amplification mixture. Amplified product was detected by enzyme immunoassay using the SK102 probe.

Haplotype determination

HLA antigens were determined by the tissue typing laboratory at Maryland Medical Laboratories (Baltimore, MD), using standard serologic assays.

HIV antibody

HIV antibody was measured with a commercial solid phase enzyme immunoassay (ELISA, Abbot Diagnostic, Abbot Park, IL).

Results

HIV specific cytotoxic T lymphocyte responses

Env-specific CTL responses were evaluated in 20 HIV-seronegative, PCR negative HCW with exposure to HIV contaminated blood/body fluids, as well as in 20 health care workers with exposure to seronegative blood and seven healthy blood donors, presumably unexposed to HIV. To induce expansion of HIV-specific primed T cells, PBMC were stimulated *in vitro* with each of the 5 individual peptides or a pool of peptides for 7 days. After this stimulation, bulk cultures of PBMC were assayed for cytotoxic activity against HIV *env* peptide pulsed-EBV transformed autologous B lymphoblastoid cell lines in a 6-h-^{51}Cr-release assay. Seven of 20 individuals with known HIV exposure exhibited cytolytic activity against the peptide-pulsed targets at least once during the study (Figure 3). HIV-specific lysis was observed only in peptide-stimulated cultures and was not detected in fresh blood tested for CTL activity. Donor 1 (3A) responded to a combination of T1 and T2 peptides as

well as to P18 IIIB. Donor 2 (3B) responded to a pool of T1 and T2 peptides. Donors 3 (3C) and 4 (3D) responded to a pool of P18 MN/P18IIIB peptides or to P18 MN, respectively. Donor 5 (3E) and 7 (3G) showed specific cytolytic activity against a pool of T1, T2 and Th4.1 peptides. Significant T1-specific CTL activity was detected in the T1-stimulated cultures from donor 6 (3F). Although peptide-specific CTL responses were detected only after *in vitro* stimulation with the peptides, the reported activity does not appear to represent a primary *in vitro* response to the peptides, since no *env*-specific lysis was detected in PBMC from any of the individuals exposed to HIV-negative blood or healthy donors. A representative negative CTL assay on *env*-stimulated cultures of an individual exposed to an HIV uninfected sample is shown on Figure 3H.

To investigate the HLA restriction of these responses, CTL activity of stimulated cultures were assayed against peptide-pulsed HLA class I-mismatched targets in some individual cases (for example, donor 2) (Figure 4A.) No HIV-specific cytolytic activity of *env*-stimulated cultures was observed against heterologous EBV-transformed mismatched targets pulsed with the stimulatory peptide. In addition, there was complete inhibition of *env*-specific lysis of autologous peptide-pulsed targets in the presence of the anti-class I monoclonal antibody (W6/32) (donor 4) (Figure 4B). The W6/32 reagent did not exhibit any inhibitory effects on antigen-specific MHC class II restricted responses.

No clear relationship emerged from the comparison between the *env*-specific CTL responses and the HLA haplotype of the CTL responders. Nevertheless, based on a previous study (M. Clerici et al., J. Immunol. 146:2214-2219 (1991)), it is noteworthy that three of the CTL responders shared HLA-A2 and all three recognized the T1/T2 pool of peptides.

The lack of cytolytic activity of

peptide-stimulated cultures on RPMI-pulsed targets, in addition to the fact that CTL responses to *env* peptides were observed upon stimulation with only certain peptides and not others, suggest that these responses are peptide-specific (Figure 5). Thus, PBMC from the donor in Figure 5A responded to P18 MN but not any of the other four peptides used individually as stimulators, despite the fact that the effectors were assayed on autologous targets pulsed with a pool of all five peptides. The PBMC from the donor in Figure 5B were stimulated and assayed on targets pulsed with the same peptides used for stimulation. This experiment demonstrates that this donor responded to P18 MN and/or P18 IIIB but not to the pool of T1/T2/Th4.1 peptides.

Temporal analysis of CTL responses

The analysis of CTL responses performed at different time points after exposure to HIV indicated variability with time among the different individuals tested (Table 6). In one individual (donor 6), CTL responses against *env* peptides were observed 1 week after exposure to HIV and no cytolytic activity from 7-day peptide-stimulated cultures were observed 69 days after exposure. In donor 1, *env*-specific responses were detected 56 days after exposure, but were absent 149 days post-exposure. The CTL response studied at a single time point in donor 4 demonstrated P18 MN-specific CTL 81 days after exposure. T1, T2 and MN specific lysis was observed 94 days post-exposure in donor 3. These responses were absent 220 days after exposure. In donor 2, CTL responses to T1T2 peptide pool were seen 143 days after exposure to HIV, but not at previous time points, with disappearance of *env*-specific responses 227 days after exposure. Similarly, responses to T1/T2/Th4.1 peptide pool observed in donor 5 after 197 days of exposure were absent 237 days after exposure to HIV. Thus, CTL responses were seen as early as 7 days after

exposure and as late as 197 days after exposure. However, the CTL memory was lost from the peripheral circulation in each case within 237 days, and often less than 40 days after a previous positive response. It is not clear whether this loss is actual loss of memory cells or compartmentalization out of the circulation. It is also important to note that the blood samples were tested in a blinded fashion, and there was no statistically significant differences (Student's t test) between the number of times that HCW exposed to HIV-positive and HIV-negative body fluids were tested (mean=2.45±1.32 SD *versus* 1.95±1.05 SD, range between 1

Table 5. Health care worker demographic characteristics

	HIV EXPOSED (n=28)	CONTROL HIV⁻EXPOSED (n=38)
SEX		
Male	19	36
Female	9	2
RACE		
White	17	28
Black	5	8
Hispanic	2	1
Asian	4	1
AGE (Mean)	34.7	33.5
JOB CATEGORY		
Nurses	9	30
Physicians	11	2
Laboratory workers	3	3
Other	5	3
PRIOR EXPOSURES		
HIV- and unknown source	11/21	19/29
HIV⁺ source	1/21	3/29
INJURY TYPE		
Mucous membrane splash	2	0
Needle Puncture	21	29
Scratch /laceration	5	8
Wound contamination	0	1
AZT administration	10	0
HIV PCR Negative	28	ND*
HIV Ab (ELISA) Negative	28	38

* ND= Not done

TABLE 6. Longitudinal analysis of *env*-specific CTL responses

DONOR	TIME (Days)	CTL RESPONSE TO	Th RESPONSE TO
1	36	NONE	T2, Th4.1, P18MN
	56	T1/T2 POOL, P18 IIIB	T1, T2
	115	P18 IIIB	NONE
	149	NONE	T1
2	51	NONE	P18IIIB, Th4.1
	79	NONE	T1
	119	NONE	T1, T2, Th4.1
	143	T1/T2 POOL	NONE
	227	NONE	NONE
3	75	P18MN/P18IIIB POOL	Th4.1
	94	T1/T2 POOL, P18MN	P18MN
	220	NONE	T2
5	197	T1/T2/Th4.1	T2, Th4.1
	237	NONE	NONE
6 TH4.1	7	T1/T2/Th4.1/P18MN/ p18IIIB POOL	T1, T2,
	34	T1	P18MN
	69	NONE	Th4.1

TABLE 7. Frequency of HIV-*env* peptide specific cytotoxic (CTL) and T helper lymphocyte responses detected in the PBMC of HCW exposed to HIV contaminated body fluids

	HIV+ FLUID EXPOSED	HIV- FLUID EXPOSED	UNEXPOSED
TH^+CTL^+	7/20 (35%)	0/20 (0%)	0/7 (0%)
TH^+CTL^-	10/20(50%)	5/20 (25%)	0/7 (0%)
TH^-CTL^+	0/20 (0%)	0/20 (0%)	0/7 (0%)
TH^-CTL^-	3/20 (15%)	15/20 (75%)	7/7 (100%)

TABLE 8. *Env*-specific T helper and CTL reactivity in exposed HCW and unexposed donors

TYPE OF EXPOSURE	T HELPER$^+$	CTL$^+$
HIV$^+$ EXPOSURE	21/28 (75%)*	7/20 (35%)*
HIV$^-$ EXPOSURE	9/37 (24%)**	0/20 (0%)
UNEXPOSED	3/33 (9%)	0/7 (0%)

* Statistically significant differences between the individuals exposed to HIV$^+$ and HIV$^-$ body fluids or unexposed blood donors, p=0.0001 (Chi-square test).

**Statistically significant differences between the HCW exposed to HIV$^-$ body fluids and unexposed blood donors, p=0.0005 (Chi-square test).

and 5). Thus, the substantially higher frequency of positives in the former group was not simply an artifact of the number of times they were tested.

Relationship between CTL and T helper responses

IL-2 production in response to the *env* peptides was tested simultaneously with the CTL assays. All of the CTL responders (7/20, 35%) were T helper reactive to the HIV *env* peptides at least once during the course of the study (Table 7). A comparison between CTL responses and T helper responses at several times after exposure to HIV indicates that CTL responses can be stimulated *in vitro* in the absence of a detectable positive *env*-stimulated IL-2 response. These findings are compared in summary in Table 6, and in detail in Figure 6. In two of the cases (6A, 6E) CTL responses appear to parallel the T helper (IL-2) responses. There was concordance between the two assays at both time points for the donor shown in Figure 6E. For one time point in three of the donors (6C, 6D) and for two time points for one donor (6B) at least 30% *env*-specific lysis was observed when the IL-2 stimulation index was less than three. Conversely, only one donor exhibited strong IL-2 responses to env when CTL activity was less than 10% (6C). These results demonstrate that CTL activity to HIV peptides can occur in the absence of T helper activity. Nevertheless, in seven of the CTL responders the HIV peptides recognized as T cytolytic epitopes were also recognized as T helper epitopes, in some cases at non-concordant time points, which may reflect differences in the kinetics of CTL and T helper activity. It is also possible that some of the IL-2 was generated by virus-specific $CD8^+$ class I restricted T cells (T. Mizouchi et al., J. Immunol. 142:270-273 (1989)).

Env-specific T helper responses evaluated in a

total of 28 HIV seronegative, PCR negative HCW with HIV exposures, indicated that 21 of them (75%) showed responses to two or more of the five peptides and in certain individuals these responses were observed more than 23 weeks after exposure. Surprisingly, 24 % (9/38) of HCW with HIV negative exposures exhibited responses to the peptides in contrast to 9% (3/33) in healthy blood donors (Table 8), and <3% in more than 200 previous low risk seronegative controls (M. Clerici *et al*, unpublished observations) and the difference between the groups were statistically significant. It cannot be ruled out that some of the exposures to negative fluids were actually to fluids from HIV-infected individuals who had not yet seroconverted. In contrast to the IL-2 (T helper) data, no *env*-specific CTL activity was detected in either of the two control groups.

To further investigate the nature of the observed CTL responses we generated *env*-specific T cell lines from CD8+ cells isolated from HIV exposed individuals. Specific lysis by these T cell lines could be induced after several rounds of stimulation using *env*-pulsed irradiated autologous PBMC as antigen presenting cells. T1 and P18 MN-specific CTL recognition of autologous targets was observed after four rounds of stimulation with autologous T1 and P18 MN-pulsed PBMC, from an HIV exposed HCW (donor 6) 425 days after exposure (Figure 7). In addition, the CTL lysed autologous P18 MN-pulsed targets but not P18MN-pulsed allogeneic HLA class I mismatched targets, indicating that the cytotoxicity observed in this system is MHC restricted (7C). It is also important to note that these CTL responses were T1 or P18 MN-specific (7A, 7B), because no significant lysis was observed against other HIV *env* peptides. In contrast, we were unable to generate *env*-specific CTL lines by parallel culture, under the same conditions, using PBMC from two donors

exposed to HIV-negative blood.

In the search for a correlate of protective immunity against progression to AIDS, researchers have begun to investigate two populations of HIV^+ individuals: 1) long term survivors (J. Laurence, J. *Characteristics of long-term survivors and long-term non-progressors with HIV infection.*, in AIDS Updates. vol. 7, pp. 1-13V. T. DeVita Jr. et al., eds., c. 1994 by J. B. Lippincott Company, Philadelphia.), patients who have survived several years, despite a diagnosis of AIDS and a low CD4 count; and 2) long-term non-progressors (J. Laurence, J. *Characteristics of long-term survivors and long-term non-progressors with HIV infection.*, in AIDS Updates. vol. 7, pp. 1-13V. T. DeVita Jr. et al., eds., c. 1994 by J. B. Lippincott Company, Philadelphia; J. Levy, J. AIDS 11:1401-1410 (1993); S.P. Buchbinder et al., AIDS 8:1123-1128 (1994)), patients who appear to be healthy and have not exhibited a decline in $CD4^+$ T cell numbers during several years of follow-up. Another potentially useful population to study are individuals who have no evidence of infection, despite multiples exposures to HIV. In contrast to the objective of the studies of survivors and non-progressors which is to investigate mechanisms of survival after infection, this latter population can be used in the search for a correlate of protective immunity against HIV infection. At least 10 publications have reported detecting T cell responses in seronegative individuals including gay men (M. Clerici et al., J. Infect. Dis. 164:178-182 (1991); M. Clerici et al., J. Infect. Dis. 165:1012-1019 (1992)), discordant sexual couples (A.M. Ranki et al., AIDS 3:83-89 (1989); H.C. Keller et al., AIDS Res. Hum. Retrovir. 8:1355-1359 (1992); P. Langlade-Demoyen et al., J. Clin. Invest. 93:1293-1297 (1994)), newborns of HIV-infected mothers (R. Cheynier et al., Eur. J. Immunol. 22:2211-2217 (1992); S.L.

Rowland-Jones et al., Lancet 341:860-861 (1993); A. DeMaria et al., J. Infect. Dis. 170:1296-1299 (1994); M. Clerici et al., AIDS 7:1427-1433 (1993)) and accidentally exposed HCW (M. Clerici et al., JAMA 271:42-46 (1994)).

The present study extends the preliminary HCW report by Clerici et al., which tested T helper cell responses in eight exposed HCW and nine controls, to now include 28 HIV-exposed seronegative HCW and 38 controls, and importantly also tests for *env*-specific CTL, as well for T helper responses. CTL activity is important to test because it suggests live virus infection of cells to get class I MHC presentation, whereas T helper cell responses could be due to exposure to dead virus. Thus, we demonstrate HIV *env*-specific CTL responses in 35% of HCW exposed to HIV contaminated body fluids and without evidence of infection by ELISA and PCR. The lack of recognition of peptide-pulsed HLA-mismatched EBV-transformed targets along with the demonstration of the blocking effect of an anti-MHC class I monoclonal antibody (W6/32) indicate that these responses were MHC class I restricted. MHC class I restricted CTL have been demonstrated to have potent anti-viral activity both *in vitro* and *in vivo*. (F. Plata et al., Cytotoxic T lymphocytes in HIV-induced disease: Implications for therapy and vaccination,pp. 401-417 in Immunodeficiencies, F. S Rosen and M. Seligmann eds., c. 1993 by Harwood Academic Publishers, New York; S.A. Kalams et al., Clinics in Laboratory Medicine 14:271-299 (1994), A. McMichael and B. D. Walker, AIDS S155-S174 (1994); R.M. Zinkernagel et al., Adv. Immunol. 27:51-177 (1979)). The alternative possibility that HIV peptides have stimulated cross-reactive CTL that were primed *in vivo* to an unrelated antigen seems unlikely because HIV-specific CTL responses were consistently not detected in the

control groups stimulated similarly. The detection of MHC class I restricted CTL indicates introduction of the HIV antigens into the endogenous antigen presentation pathway. Induction of virus-specific CTL usually requires *in vivo* priming with infectious viruses. Inactivated virus, viral proteins and peptides are in most cases ineffective for *in vivo* induction of class I restricted CTL, although such antigen preparations can induce antibody responses to viral proteins (K. Deres et al., Nature 342:561-564 (1989); G.L. Ada et al., Curr. Top. Microb. Immunol. 128:1-54 (1986)). Class I restricted T cells generally recognize antigens that are synthesized within an antigen-presenting cell (APC) or that otherwise enter the cytosol of the APC (L.A. Morrison et al., J. Exp. Med. 163:903-921 (1986); R.N. Germain, Nature 322:687-689 (1986); M.W. Moore et al., Cell 54:777-785 (1988); J. Neefjes et al., Cell 61:171-183 (1991)). It is conceivable therefore that a low level of infection occurred but was contained by cell mediated immune mechanisms , and was undetectable by PCR performed in the peripheral blood. The possibility of occurrence of HIV sequences in lymphoid tissues could not be addressed in this study. Although the mechanisms of *in vivo* induction of CTLs are incompletely understood, alternative explanations for the observed cell mediated immune responses to HIV include primary *in vivo* stimulation of CD8+ cells with defective viral particles that might gain access to the cytosol, or might bind to MHC class I molecules of cells with alternate pathways of antigen presentation by MHC class I involving endolysosomal processing of antigens (B. Pernis Immunol. Today. 6:45-49 (1985); J.H. Hochman et al., J. Immunol. 146:1862-867 (1991)). Recent reports have demonstrated that peripheral blood dendritic cells are able to prime naive CD8+ cells to soluble antigens, including HIV peptides, leading to

the generation of potent antigen-specific CTL (S.E. Macatonia et al., J. Exp. Med. 169:1255-1264 (1989); A. Mehta-Damani et al., J. Immunol. 153:996-1003 (1994)). In addition, a recent report indicates that intravenous infusion of HIV peptide-pulsed murine dendritic cells can induce HIV-specific CD8+ cells *in vivo* (H. Takahashi et al., Int. Immunol. 5:849-857 (1993)). However, the possibility that this mechanism explains our findings seems unlikely based on the fact that dendritic cells represent 1 to 2% of circulating peripheral blood mononuclear cells and the volume of blood transferred was very small.

Two aspects of our data also suggest that we restimulated a recall response, rather than priming a CTL response from a previously naive population *in vitro*. First, specific CTL activity was observed only in *env*--stimulated cultures from HIV exposed individuals (7/20) and not in those exposed to HIV negative samples (0/20) or healthy blood donors (0/7). Second, a single round of peptide stimulation was sufficient to detect cytolytic activity from bulk cultures, suggesting the presence of HIV-specific CTL memory precursors in circulation in these individuals. In contrast, the *in vitro* primary response described required multiple stimulations (S.E. Macatonia et al., J. Exp. Med. 169:1255-1264 (1989); A. Mehta-Damani et al., J. Immunol. 153:996-1003 (1994)). Furthermore, we were unable to demonstrate CTL activity from fresh PBMC. This contrasts with the observation that HIV-specific CTL against several HIV antigens can be detected in fresh PBMC from many HIV-infected individuals (B.D. Walker et al., Nature 328:345-348 (1987); B.D. Walker et al., Science 240:64-66 (1988)); R.A. Koup et al., Blood 73:1909-1914 (1989); F. Buseyne et al., J. Virol. 67:694-702 (1993)).

Several lines of evidence suggest that CTL are an important component of the immune response to HIV

infection. HIV-specific CTL precursors are present at high frequency very early during infection, often detected before seroconversion (6). During the subsequent prolonged asymptomatic phase of infection, HIV specific $CD8^+$ MHC class I restricted CTL activity can be detected directly from peripheral blood in the absence of *in vitro* stimulation (B.D. Walker et al., Nature 328:345-348 (1987); B.D. Walker et al., Science 240:64-66 (1988)); R.A. Koup et al., Blood 73:1909-1914 (1989); F. Buseyne et al., J. Virol. 67:694-702 (1993)). Progression to AIDS is accompanied by an increase in virus replication and a loss of $CD8^+$ HIV-specific CTL activity (A. Carmichael et al., J. Exp. Med. 177:249-256 (1993), R.I. Connor et al., J. Virol. 67:1772-1777 (1993)). The association of the CTL response with the initial acute loss of viremia (R.A. Koup et al., J. Virol. 68:4650-4655 (1994)) and the subsequent loss of that control with progression to AIDS suggests that the CTL response contributes to the control of HIV replication during the asymptomatic phase of infection. Furthermore, studies on long-term non-progressors demonstrated that a vigorous and broadly reactive CTL response can be detected in serpositive persons with normal CD4 counts who have been infected for up to 15 years (A. McMichael and B. D. Walker, AIDS S155-S174 (1994); T.C. Greenough et al., AIDS Res. Human Retrovir. 10:395-403 (1994)). The presence of MHC class I restricted CTL responses has also been detected in multiply sexually or perinatally exposed, HIV seronegative individuals who remain virus-free by PCR (R. Cheynier et al., Eur. J. Immunol. 22:2211-2217 (1992); S.L. Rowland-Jones et al., Lancet 341:860-861 (1993); A. DeMaria et al., J. Infect. Dis. 170:1296-1299 (1994); P. Langlade-Demoyen et al., J. Clin. Invest. 93:1293-1297 (1994)). It can be argued that these responses may have conferred resistance to HIV infection in the absence of antibody

in the HIV exposed individuals without evidence for infection based on antibody production and PCR.

The variability of the kinetics of CTL responses might reflect differences in the CTL precursors in circulation between different individuals at different time points. Factors present at the moment of exposure, such as host's genetic susceptibility, inoculum type (defective or avirulent virus), inoculum size and pathogenicity of viral strain, as well as the host's ability to elicit a protective immune response could determine the outcome of the encounter with HIV. This possibility is supported by recent animal experiments, in which vaginal innoculation with less virulent simian immunodeficiency virus (SIV) appeared to result in transient detection of virus without persistent infection and development of low but detectable immune responses (M.L. Marthas et al., J. Med. Primatol. 21:99-107 (1992)). Further support for this possibility comes from a study demonstrating resistance to SIV challenge in macaques previously exposed intravenously to sub infectious doses of SIV (M. Clerici et al., AIDS 8:1391-1395 (1994)). The host's prior immunological background might also influence the immune system's ability to handle unrelated heterologous viral infections, as recently reported in murine viral infections (L.K. Selin et al., J. Exp. Med. 179:1933-1943 (1994); L.K. Selin and R. M. Welsh, Current Opinion in Immunology 6:553-559 (1994)).

CTL responses were observed in the absence of simultaneous T helper reactivity to the *env* peptides, as measured by IL-2 production in response to the peptides. The apparent dichotomy between CTL and T helper reactivity is also in agreement with studies in which CTL responses could be induced in the absence of $CD4^+$ T cell help (Y. Wu Y. Liu, Current Biology 4:499-505 (1994)), and with the observation of intact

$CD8^+$ CTL function in CD4-deficient knockout mice (A. Rahemtulla et al., Nature 353:180-184 (1991)). Furthermore, recent studies on IL-2 negative mice suggested that this factor is not essential for either NK cell and or CTL activation during viral infection of mice (T.M. Kundig et al., Science 262:1059-1061 (1993); C.A. Biron, Current Opinion in Immunology 6:530-538 (1994)). It is very possible that T helper responses were present, with specificity for epitopes different from those that we tested.

Our finding of coincident *env*-specific T helper and CTL responses to the same peptides raises the possibility that we selected responses from individuals whose T cells responded to particular peptides for both helper and effector function, since the cultures were not supplemented with exogenous IL-2. Alternatively, some IL-2 could have been produced by $CD8^+$ T cells, as was reported for murine T cell responses from mice infected with vaccinia virus (T. Mizouchi et al., J. Immunol. 142:270-273 (1989)).

T helper responses were detected in a much higher proportion of HIV exposed HCW than CTL responses (75% versus 35%), which is consistent with promiscuous T cell recognition of the peptides in the contest of several MHC class II alleles (R.M. Chicz et al., J. Exp. Med. 178:27-47 (1993)). T helper but not CTL responses were observed in 24% of the control group exposed to seronegative blood. This high " background" could be accounted for by the putative cross-reactivity between HIV *env* and other antigens to which these individuals were previously exposed, particularly the alloantigens to which the control groups of HCW were exposed. This possibility is consistent with the reported homologies between HIV proteins and self antigens (J.L. Ziegler and D.P. Stites, Clin. Immunol. Immunopathol. 41:305-313 (1986); J.M. Andrieu et al., J. Acq. Immune Defic.

Syndr. 2:163-174 (1986); J.A.T. Young, Nature 333:215 (1988); H. Golding et al., J. Exp. Med. 167:914-923 (1988); E.F. Hounsell et al., Mol. Asp. of Med. 12:283-296 (1991); M. Clerici et al., Eur. J Immunol. 23:2022-2025 (1993)). However, since the fraction positive is significantly higher than in other control groups, it is possible that some of the fluids were infected even though the donor was seronegative (i.e., had not yet seroconverted).

The ability to generate HIV *env* -specific $CD8^+$ cell lines more than one year after HIV exposure favors the possibility that low dose exposure to HIV might generate HIV-specific long-term memory CTL precursors that could protect against subsequent exposure to HIV, as has been reported in animal models of other viral infections (P.C. Doherty et al., Current Opinion in Immunology 6:545-552 (1994); L.L. Lau et al., Nature 369:648-652 (1994); A. Mullbacher, J. Exp. Med. 179:317-321 (1994)).

The demonstration of CTL responses along with the observation of T helper responses to HIV peptides in HIV exposed but uninfected health care workers indicate that *in vivo* priming of T helper and cytotoxic T lymphocytes can occur after single documented accidental occupational exposure to HIV, and raises the possibility that cell mediated immune mechanisms will be involved in the containment of HIV infection.

The invention being thus described, it will be clear to one skilled in the art that particular materials and methods may be varied in ways obvious to such a skilled practitioner. As such, the detailed experiments set forth in the Examples are meant to be illustrative, rather than limiting, of the scope of the invention as claimed herein below.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Gov't. of the United States as represented by the Department of Health and Human Services/National Institutes of Health, Office of Technology Transfer
(B) STREET: 6011 Executive Blvd.
(C) CITY: Rockville
(D) STATE OR PROVINCE: Maryland
(E) COUNTRY: United States of America
(F) POSTAL CODE: 20852

(ii) TITLE OF INVENTION: TEST OF HIV-SPECIFIC T LYMPHOCYTE FUNCTION THAT DETECTS EXPOSURE TO HIV ANTIGENS AND POSSIBLY EARLY HIV INFECTION

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Birch, Stewart, Kolash & Birch
(B) STREET: 301 N. Washington
(C) CITY: Falls Church
(D) STATE: Virginia
(E) COUNTRY: USA
(F) ZIP: 22046-0747

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Svensson, Leonard R.
(B) REGISTRATION NUMBER: 30,330
(C) REFERENCE/DOCKET NUMBER: 1173-501P

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 703-241-1300
(B) TELEFAX: 703-241-2848

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1
(B) STRAIN: IIIB

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /label= Peptide_T1
/note= "synthetic peptide corresponding to residues 428-443 of the Env protein of HIV-1 isolate IIIB"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human Immunodeficiency virus type 1
(B) STRAIN: IIIB

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..13
(D) OTHER INFORMATION: /label= peptide_T2
/note= "synthetic peptide corresponding to residues 112-124 of the HIV-1 Env protein of isolate IIIB"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

His Glu Asp Ile Ile Ser Leu Trp Asp Gln Ser Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human Immunodeficiency Virus type I
(B) STRAIN: IIIB

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /label= peptide_TH4-1
/note= "synthetic peptide corresponding to residues 834-848 of the Env protein of HIV-1 isolate IIIB"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asp Arg Val Ile Glu Val Val Gln Gly Ala Tyr Arg Ala Ile Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human Immunodeficiency Virus type I
(B) STRAIN: IIIB

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /label= peptide_P18IIIb
/note= "synthetic peptide corresponding to residues 315-329 of the Env protein of HIV-1 isolate IIIb"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Ile Gln Arg Gly Pro Gly Arg Ala Phe Val Thr Ile Gly Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human Immunodeficiency Virus type I
(B) STRAIN: MN

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /label= peptide_P18MN
/note= "synthetic peptide corresponding to region homologous to P18-IIIb, but from isolate MN of HIV-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human Immunodeficiency Virus type I

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /label= peptide_P23
/note= "Non-immunogenic synthetic peptide from HIV-1 Env protein, corresponds to residues 369-383"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Lys Gln Ser Ser Gly Gly Asp Pro Glu Ile Val Thr His Ser Phe
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Sperm whale

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /label= Peptide_myo
/note= "Immunogenic, non-HIV syntetic peptide homologous to residues 132-146 of sperm whale myoglobin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Lys Ala Leu Glu Leu Phe Arg Lys Asp Ile Ala Ala Lys Tyr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human Immunodeficiency Virus

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /label= oligonucleotide
/note= "synthetic oligonucleotide used as upstream primer in PCR diagnostic test for HIV infection. Detects nef gene product; nucleotides 9048-9061 of

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGACTTACAA GGCAGCTATA GATC 24

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human Immunodeficiency Virus

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..22
(D) OTHER INFORMATION: /label= oligonucleotide
/note= "synthetic oligonucleotide used as downstream primer in PCR diagnostic test for HIV infection. Detects nef gene product; nucleotides

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTCTGGATCA ACTGGTACTA GC 22

CLAIMS

What is claimed is:

1. A method for diagnosing exposure of a patient to an infectious agent which comprises:

a) obtaining peripheral blood mononuclear cells from a patient;

b) incubating said peripheral blood mononuclear cells with at least one synthetic peptide representing an epitope(s) of said infectious agent; and

c) determining the activation of said peripheral blood mononuclear cells as a result of the incubation in step (b).

2. A method for diagnosing exposure of a patient to an infectious agent which comprises:

a) identifying at least one peptide epitope present in antigens of said infectious agent which provoke an immune response in a mammal;

b) obtaining peripheral blood mononuclear cells from a patient;

c) incubating said peripheral blood mononuclear cells with at least one synthetic peptide representing the epitope(s) of step (a); and

d) determining the activation of said peripheral blood mononuclear cells as a result of the incubation in step (c).

3. The method of claim 1, wherein said activation is determined by measuring cytokine production by said peripheral blood mononuclear cells.

4. The method of claim 3, wherein said cytokine is interleukin-2.

5. The method of claim 1, wherein said infectious agent is a virus.

6. The method of claim 1, wherein said infectious agent is Human Immunodeficiency Virus.

7. The method of claim 2, wherein said activation is determined by measuring cytokine production by said peripheral blood mononuclear cells.

8. The method of claim 7, wherein said cytokine is interleukin-2.

9. The method of claim 2, wherein said infectious agent is a virus.

10. The method of claim 2, wherein said infectious agent is Human Immunodeficiency Virus.

11. The method of claim 3, wherein said measuring is performed by immunoassay of said cytokine.

12. The method of claim 3, wherein said measuring is performed by measuring proliferation of a cytokine-dependent cell line.

13. The method of claim 7, wherein said measuring is performed by immunoassay of said cytokine.

14. The method of claim 7, wherein said measuring is performed by measuring proliferation of a cytokine-dependent cell line.

15. The method of claim 6, wherein the peptides are chosen from the group consisting of KQIINMWQEVGKAMYA, HEDIISLWDQSLK, DRVIEVVQGAYRAIR, RIQRGPGRAFVTIGK, and RIHIGPGRAFYTTKN.

16. The method of claim 10, wherein the peptides are chosen from the group consisting of KQIINMWQEVGKAMYA, HEDIISLWDQSLK, DRVIEVVQGAYRAIR, RIQRGPGRAFVTIGK, and RIHIGPGRAFYTTKN.

17. A method according to claim 15, wherein a positive diagnosis is indicated by positive response to at least two of the peptides.

18. A method according to claim 16, wherein a postive diagnosis is indicated by positive response to at least two of the peptides.

19. A method for diagnosing exposure of a patient to an infectious agent which comprises:

a) identifying peptide epitopes present in antigens of said infectious agent which provoke an immune response in a mammal;

b) obtaining mononuclear cells from the lymph nodes or spleen of a patient;

c) incubating said mononuclear cells from step (2) with synthetic peptides representing the epitopes of step 1; and

d) determining the activation of said mononuclear cells a result of the incubation in step (3).

20. The method of claim 1, wherein killing activity of cytotoxic T lymphocytes is measured in step c.

21. The method of claim 5, wherein killing activity of cytotoxic T lymphocytes is measured in step c.

22. The method of claim 15, wherein killing activity of cytotoxic T lymphocytes is measured in step c.

23. The method of claim 19, wherein killing activity of cytotoxic T lymphocytes is measured in step d.

24. The method of claim 1, wherein proliferation of T helper lymphocytes is measured in step c.

25. The method of claim 19, wherein proliferation of T helper lymphocytes is measured in step c.

FIG. 1A

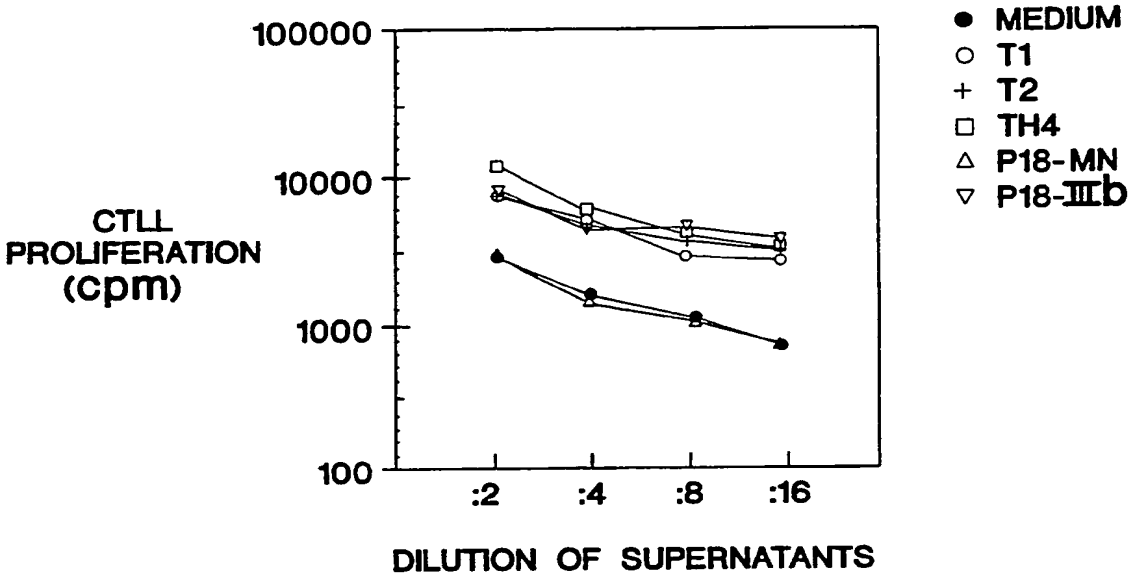


FIG. 1B

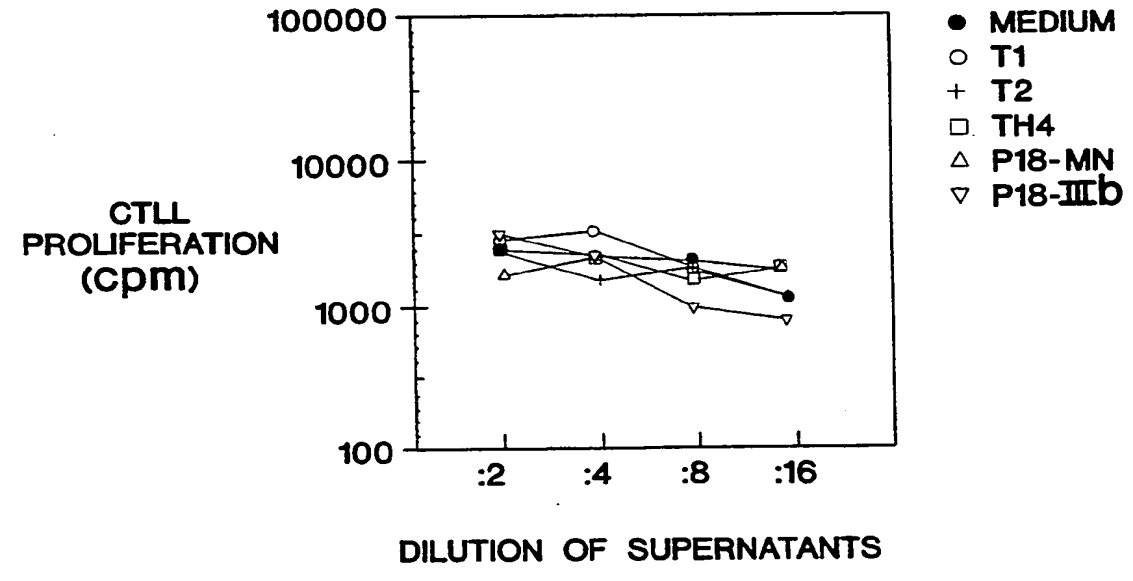


FIG. 2A(1)

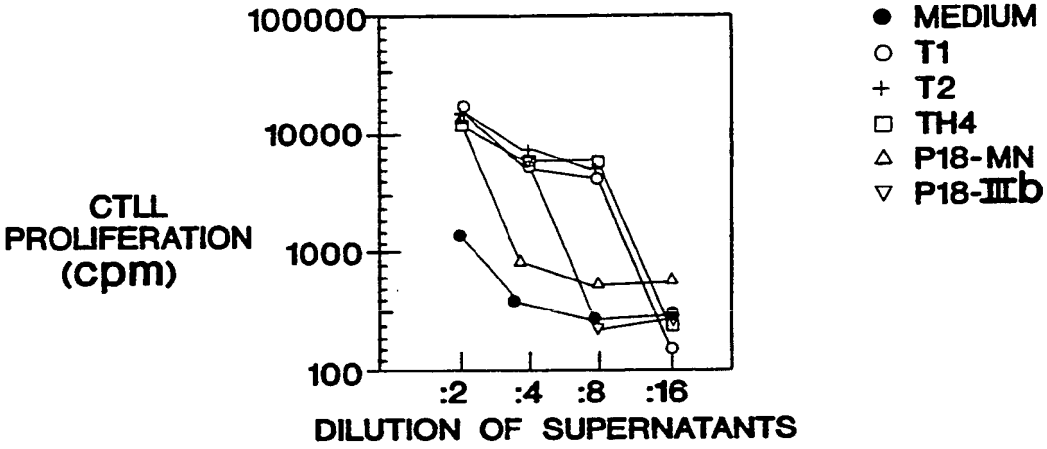


FIG. 2A(2)

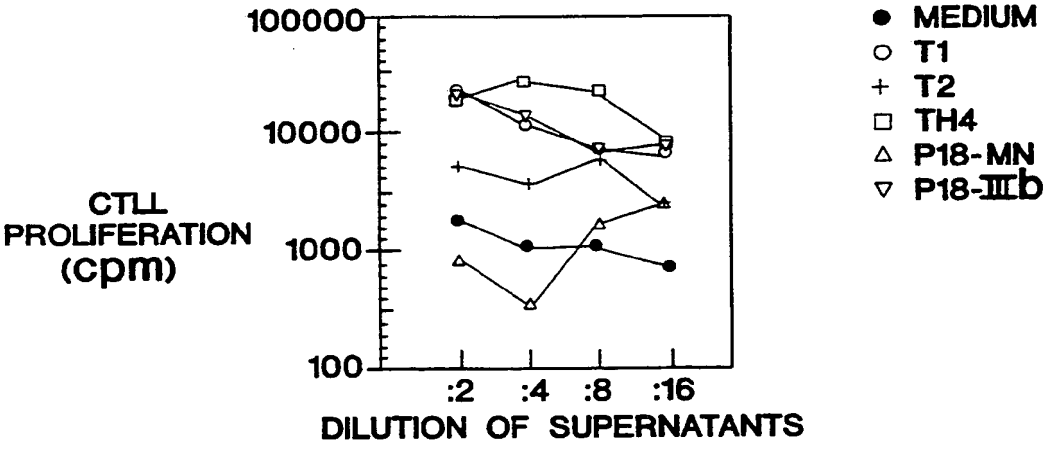


FIG. 2A(3)

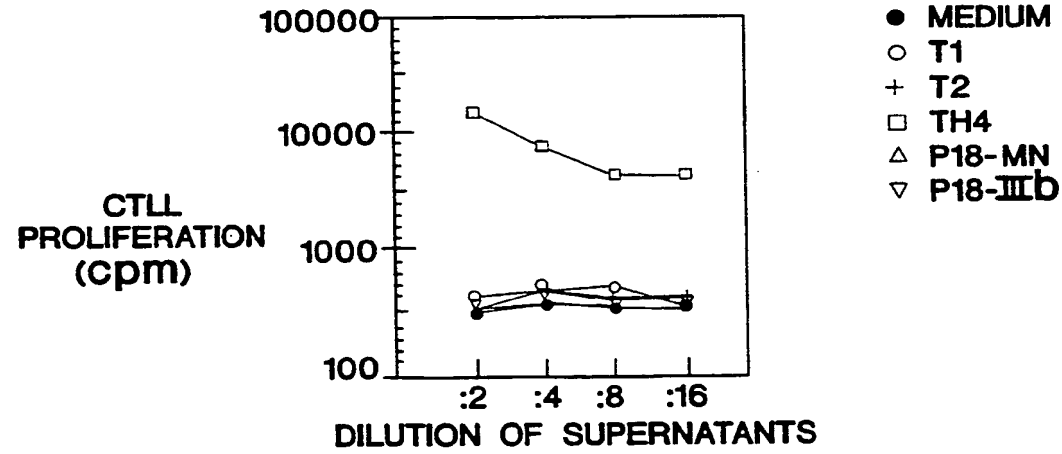


FIG. 2B(1)

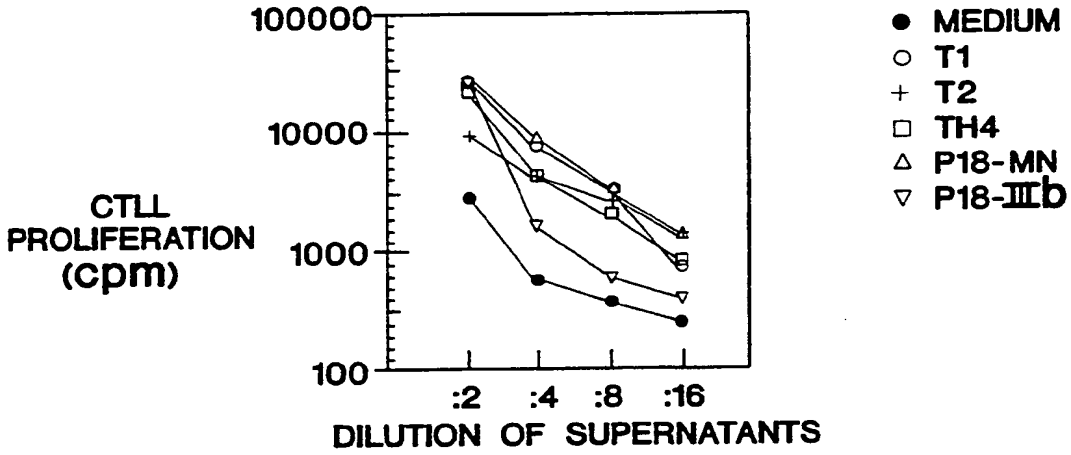


FIG. 2B(2)

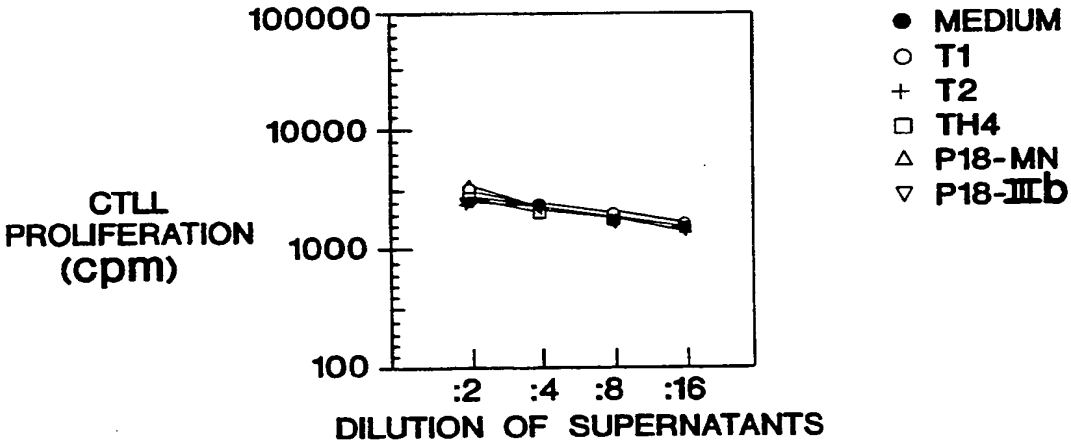


FIG. 2B(3)

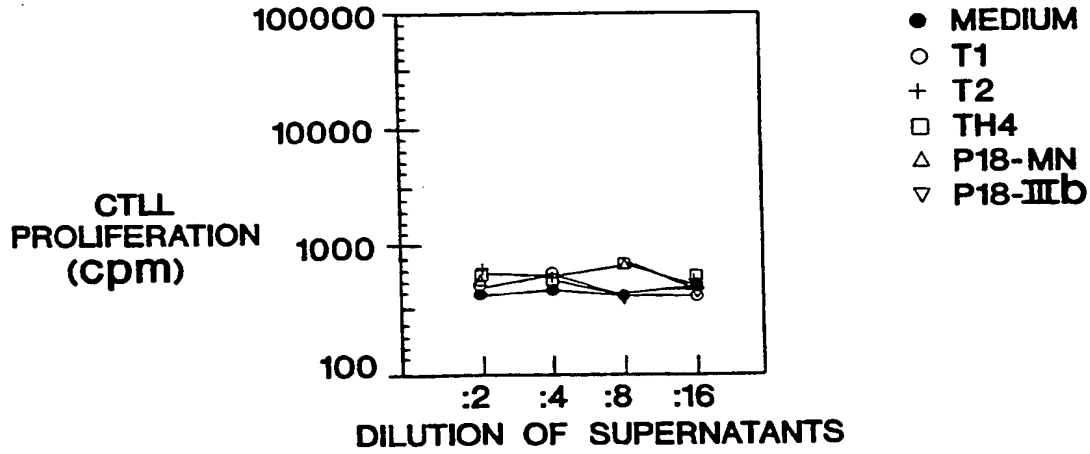


FIG. 2C(1)

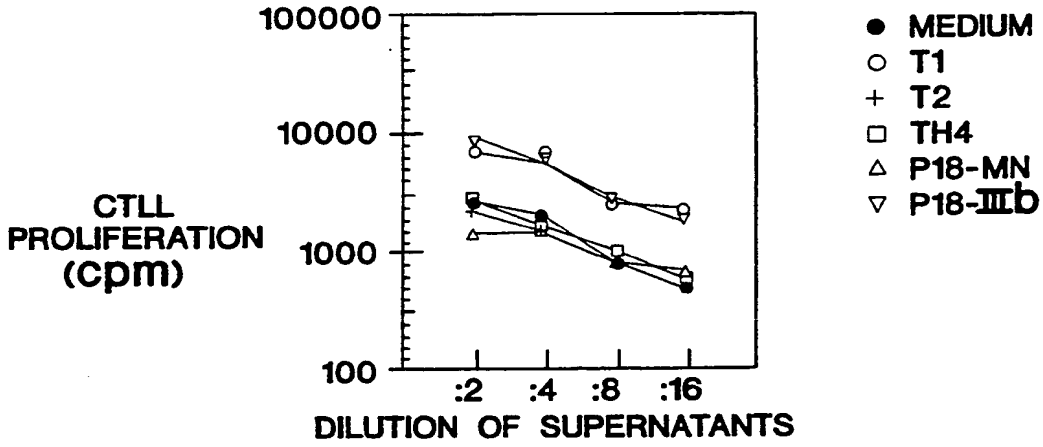


FIG. 2C(2)

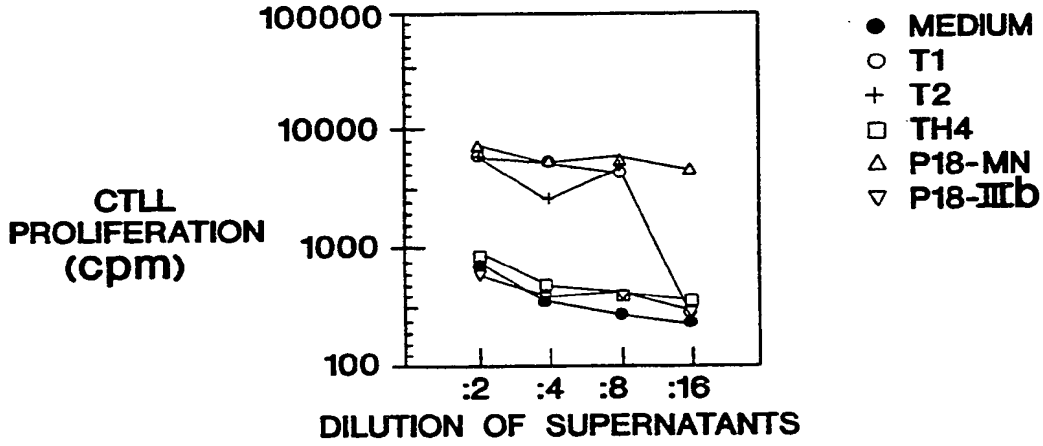


FIG. 2C(3)

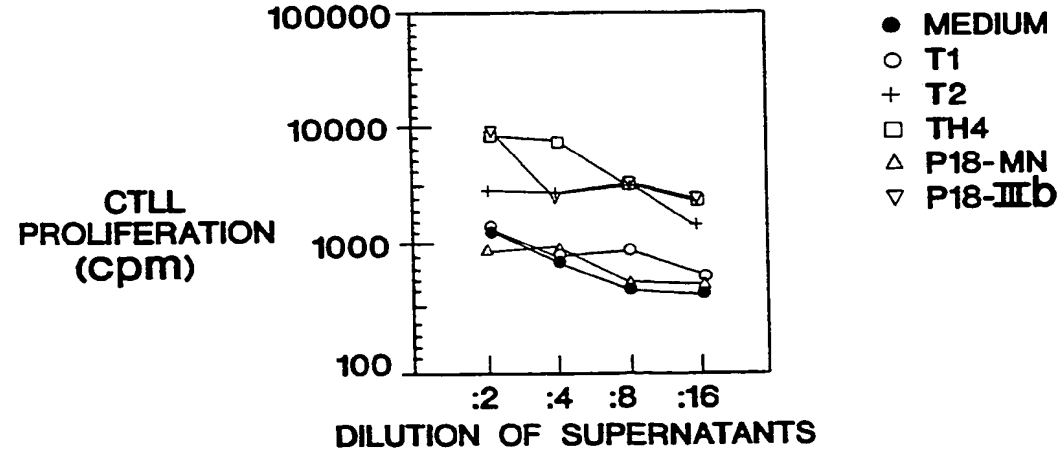


FIG. 2D(1)

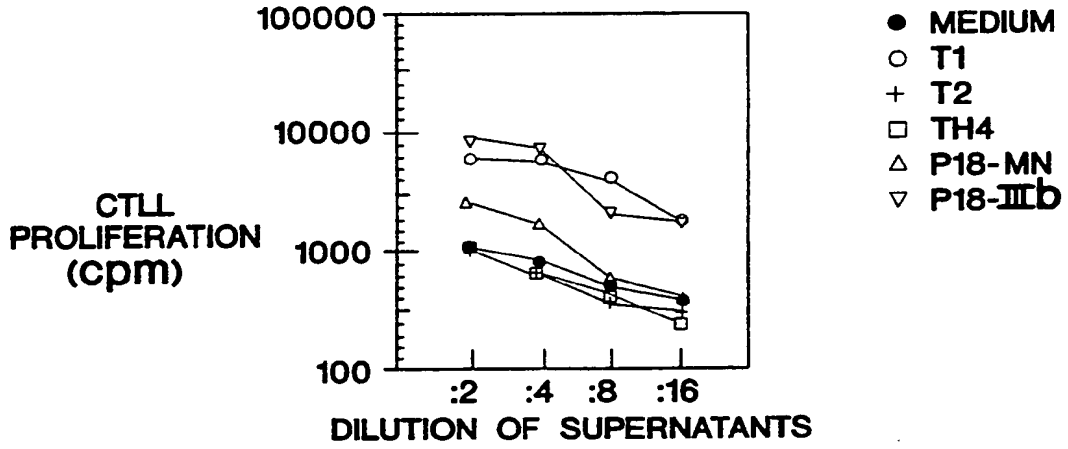


FIG. 2D(2)

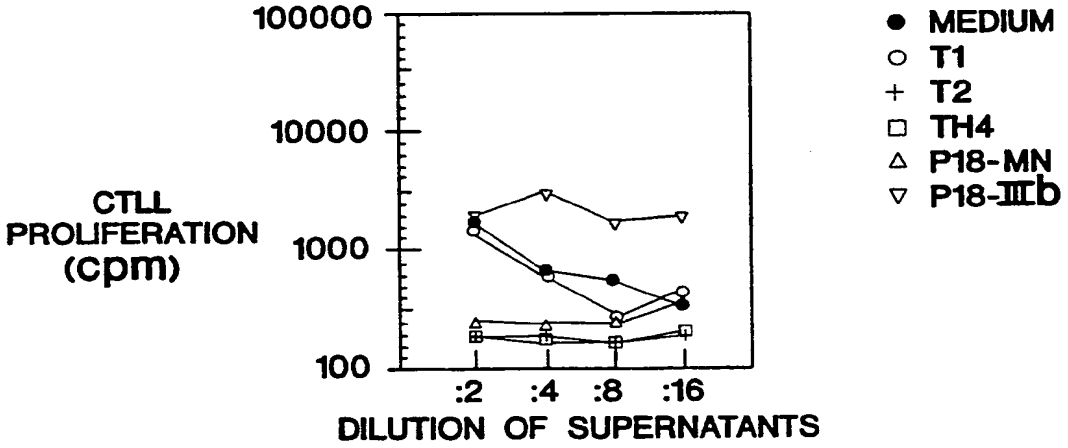


FIG. 2D(3)

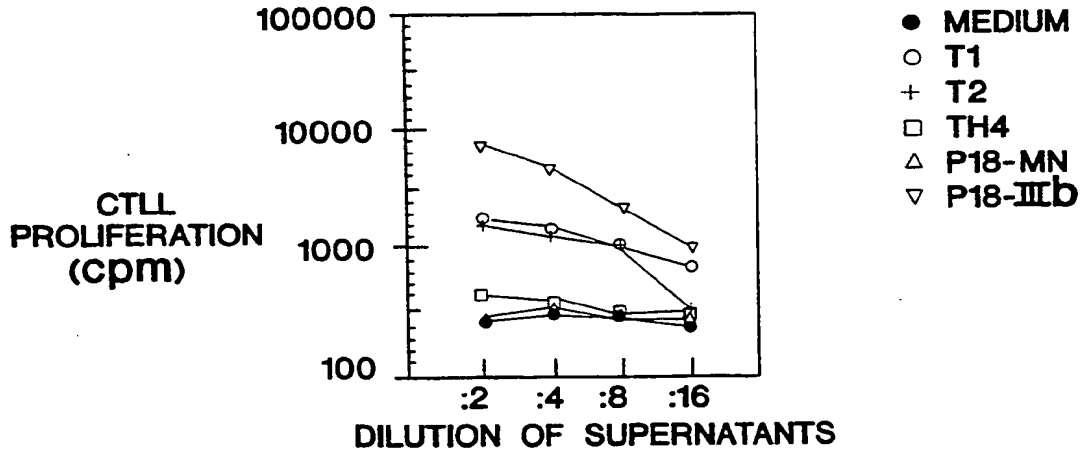


FIG. 2E(1)

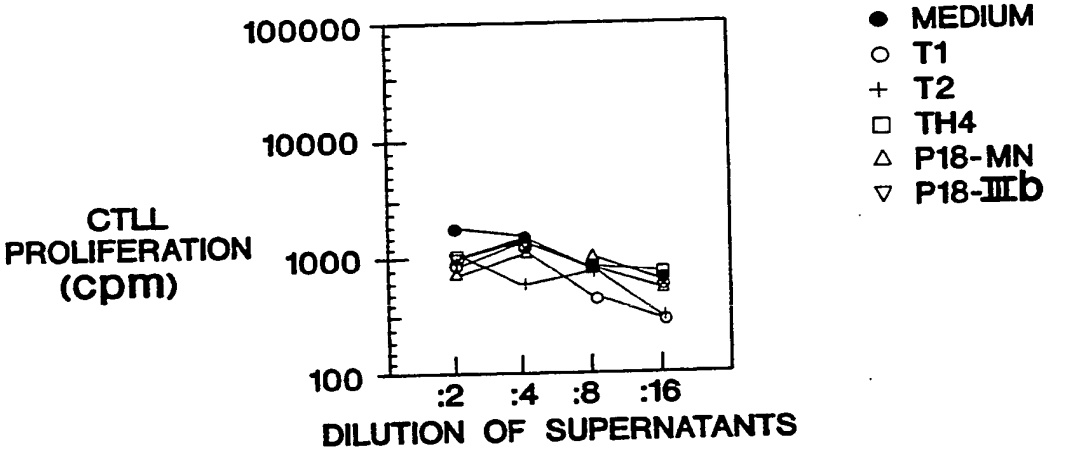


FIG. 2E(2)

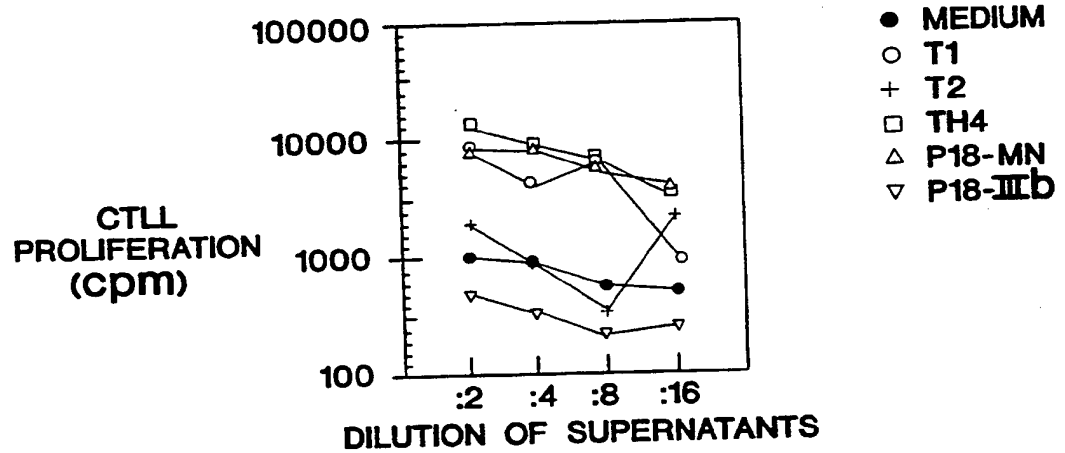


FIG. 2E(3)

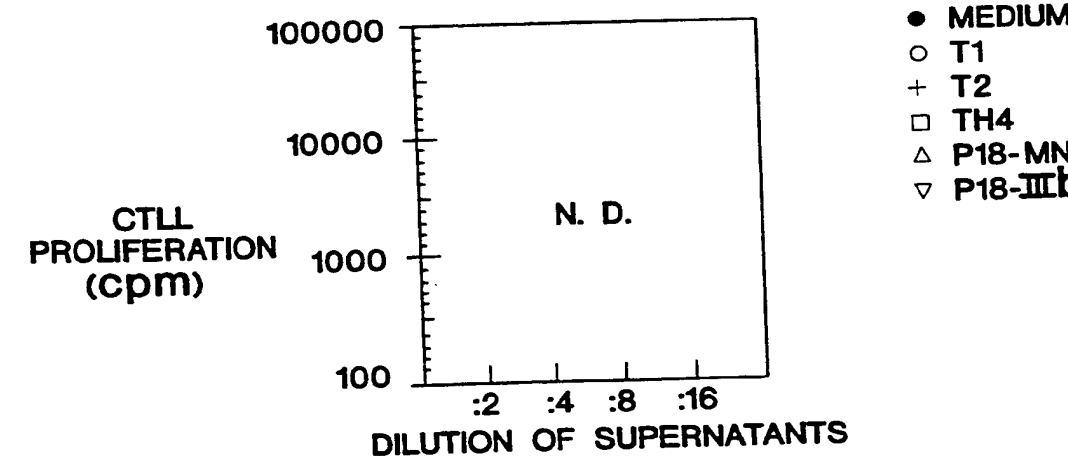


FIG. 3B

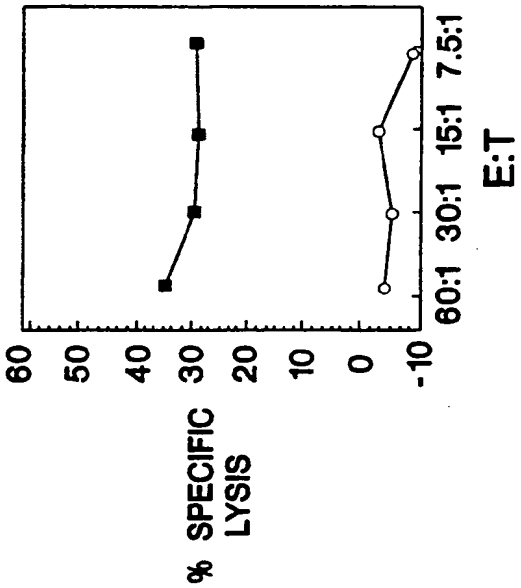


FIG. 3D

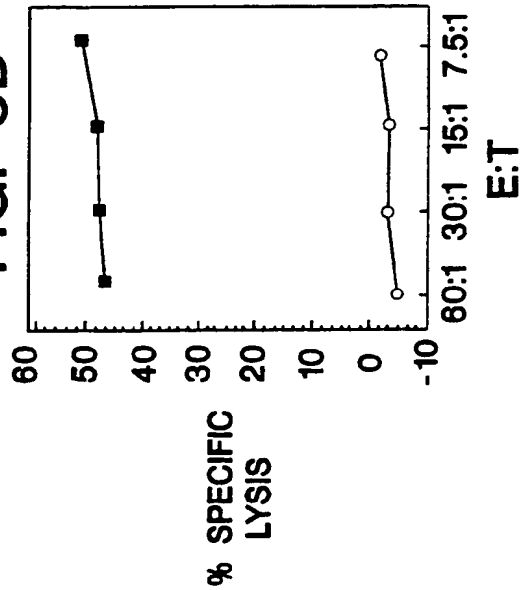


FIG. 3A

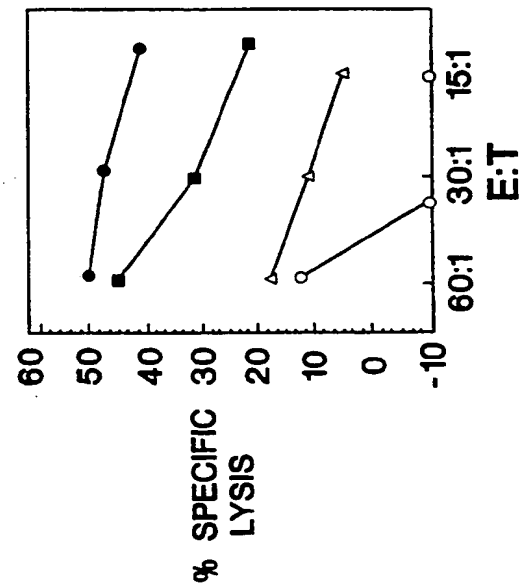


FIG. 3C

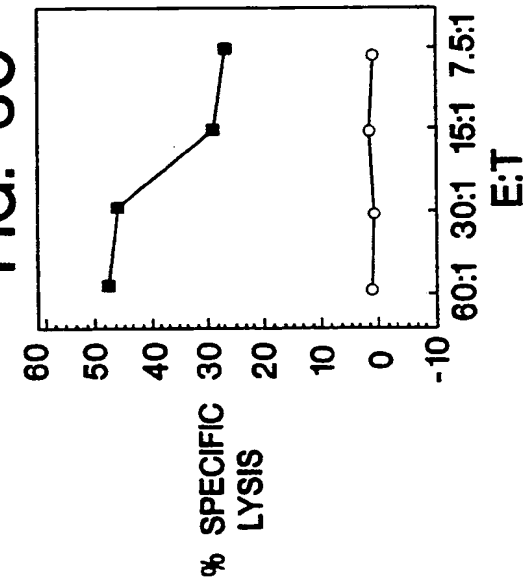


FIG. 3F

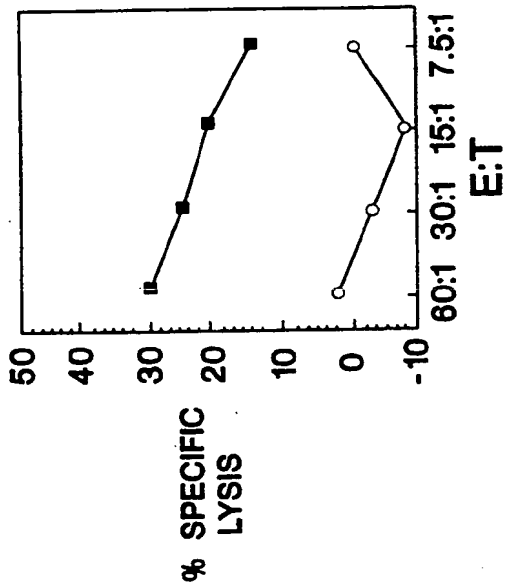


FIG. 3H

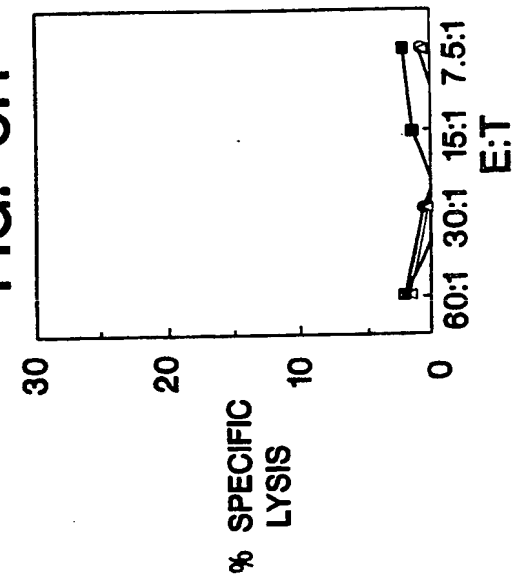


FIG. 3E

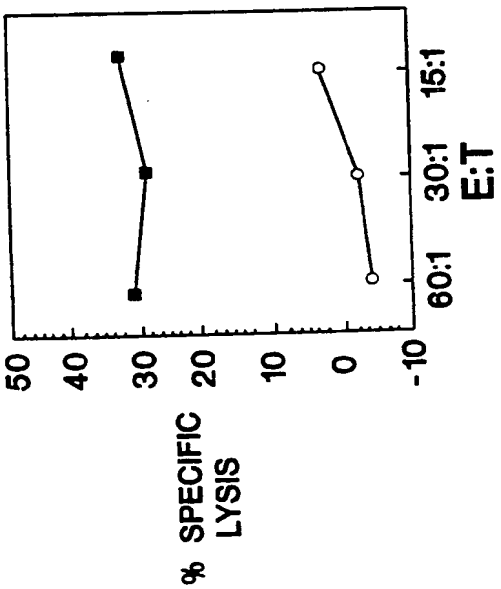


FIG. 3G

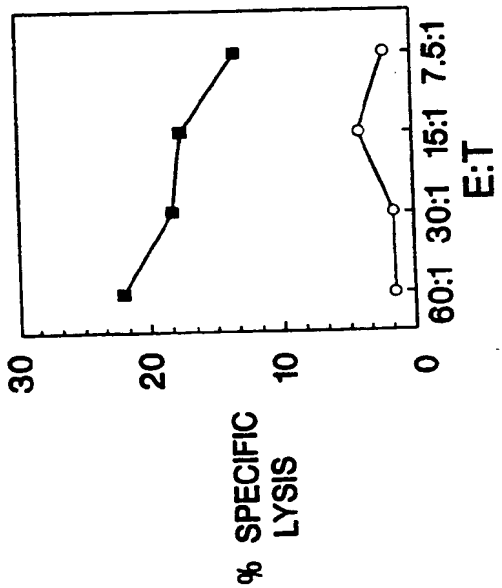


FIG. 4A

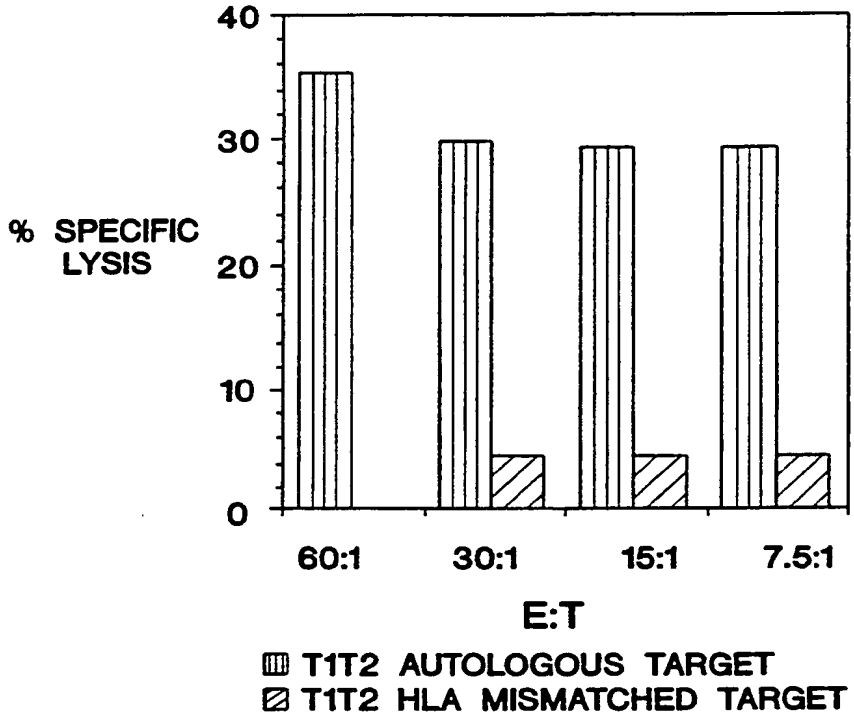


FIG. 4B

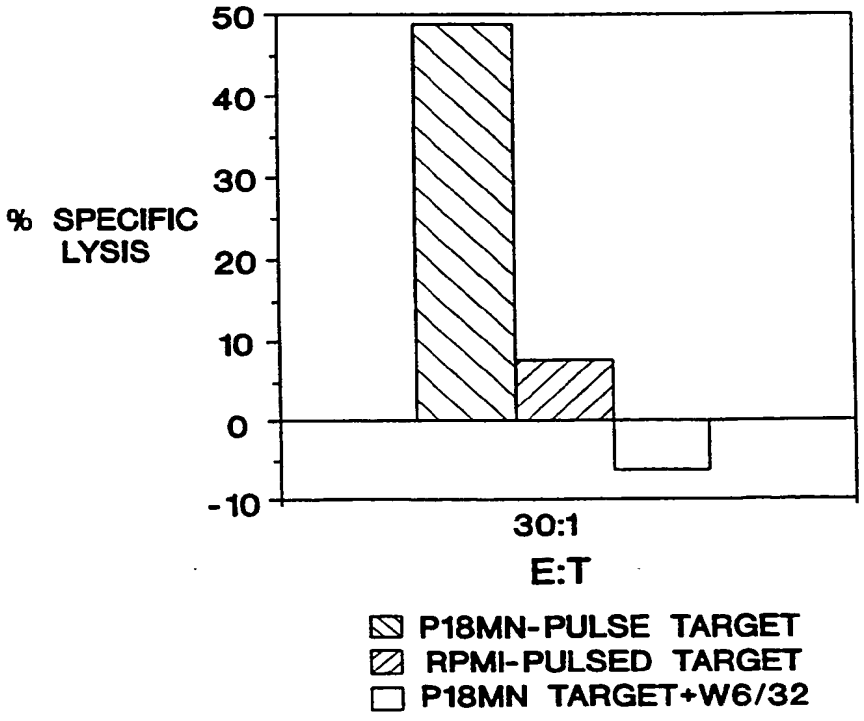


FIG. 5A

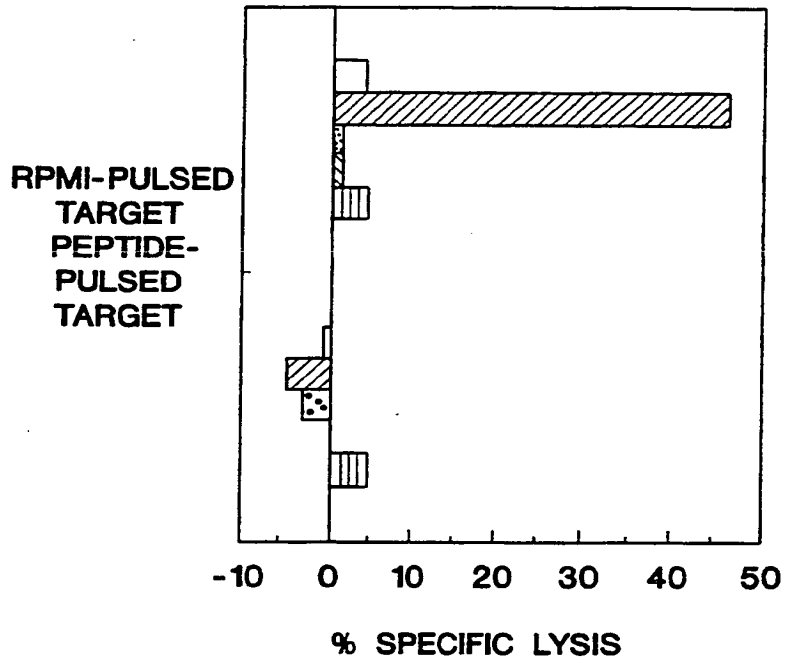


FIG. 5B

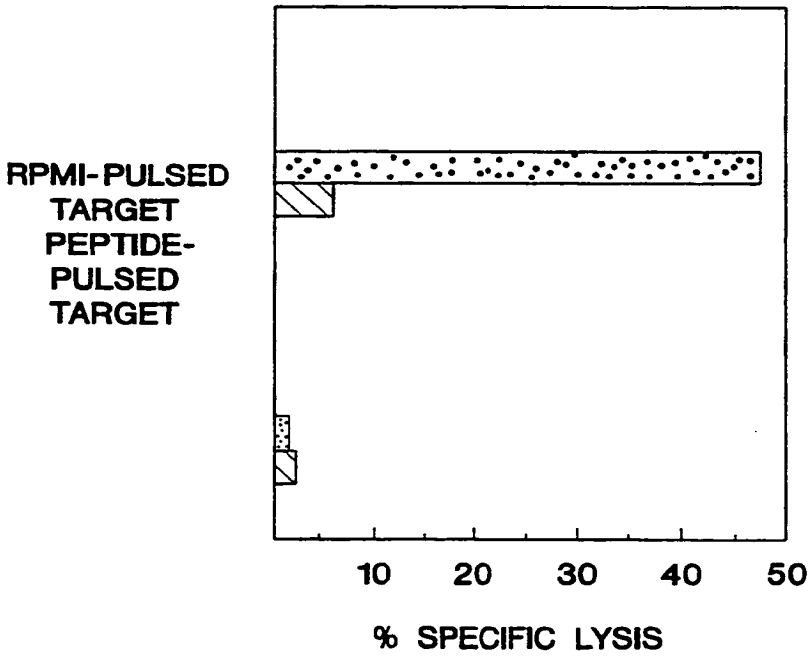


FIG. 6A

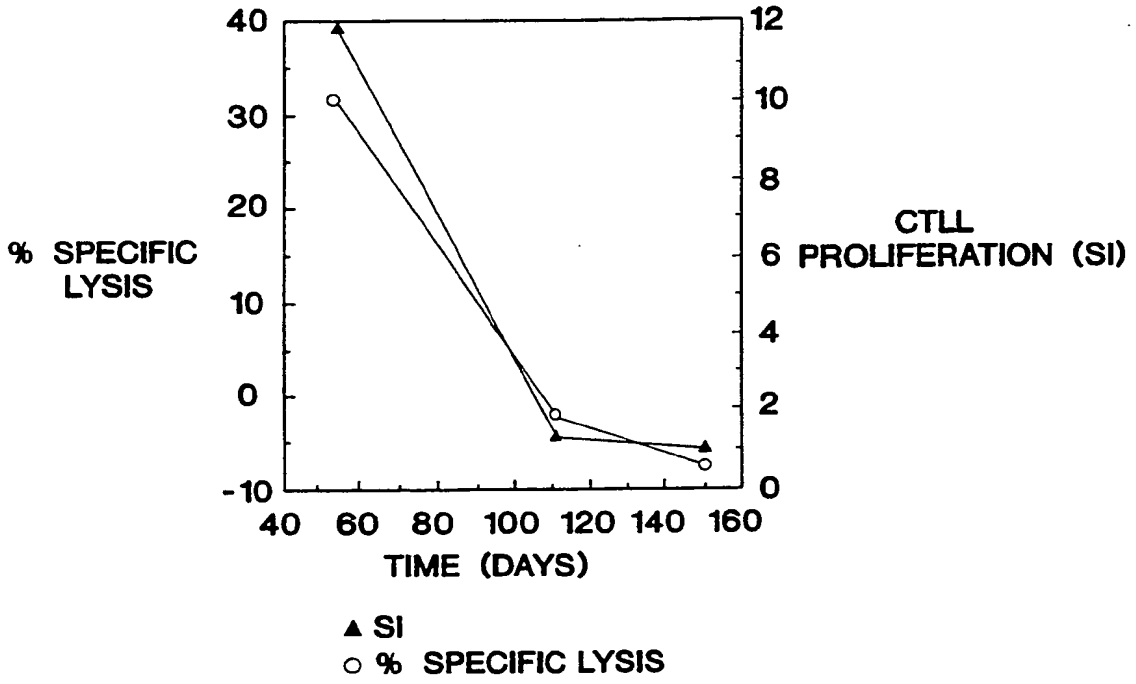


FIG. 6B

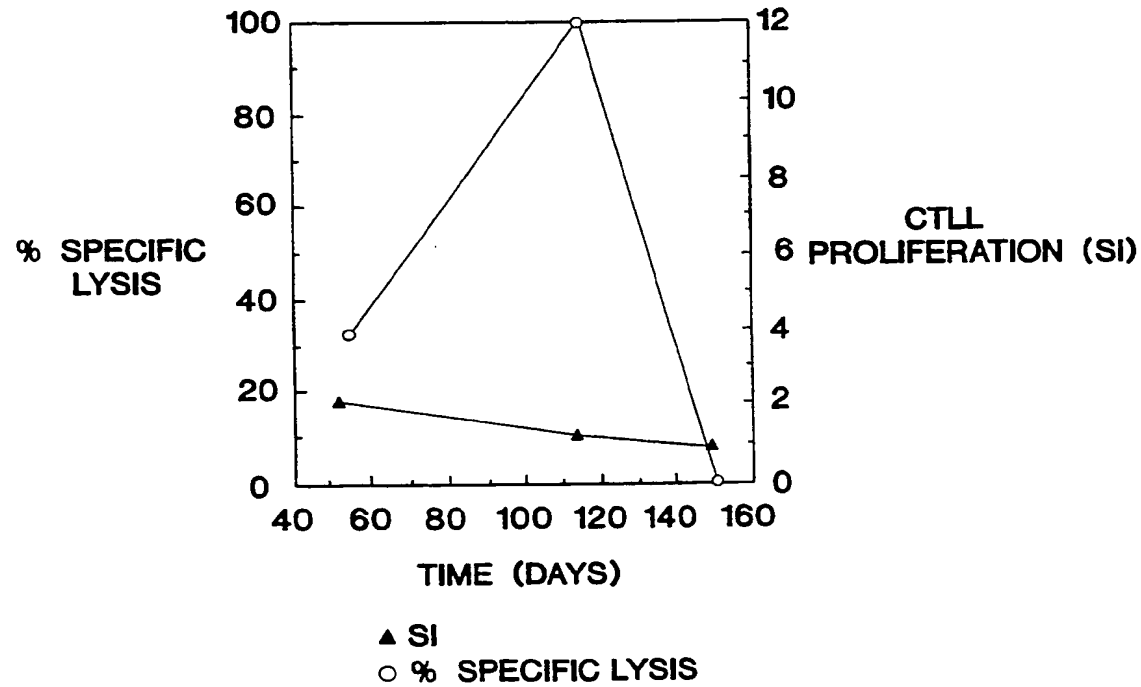


FIG. 6C

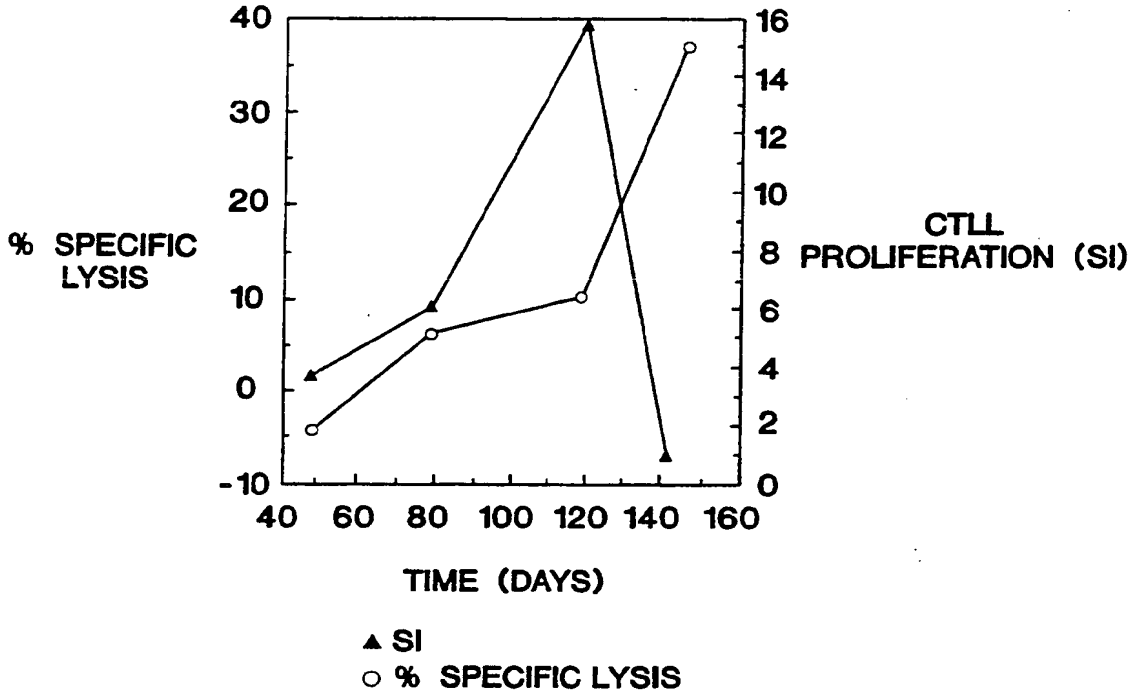


FIG. 6D

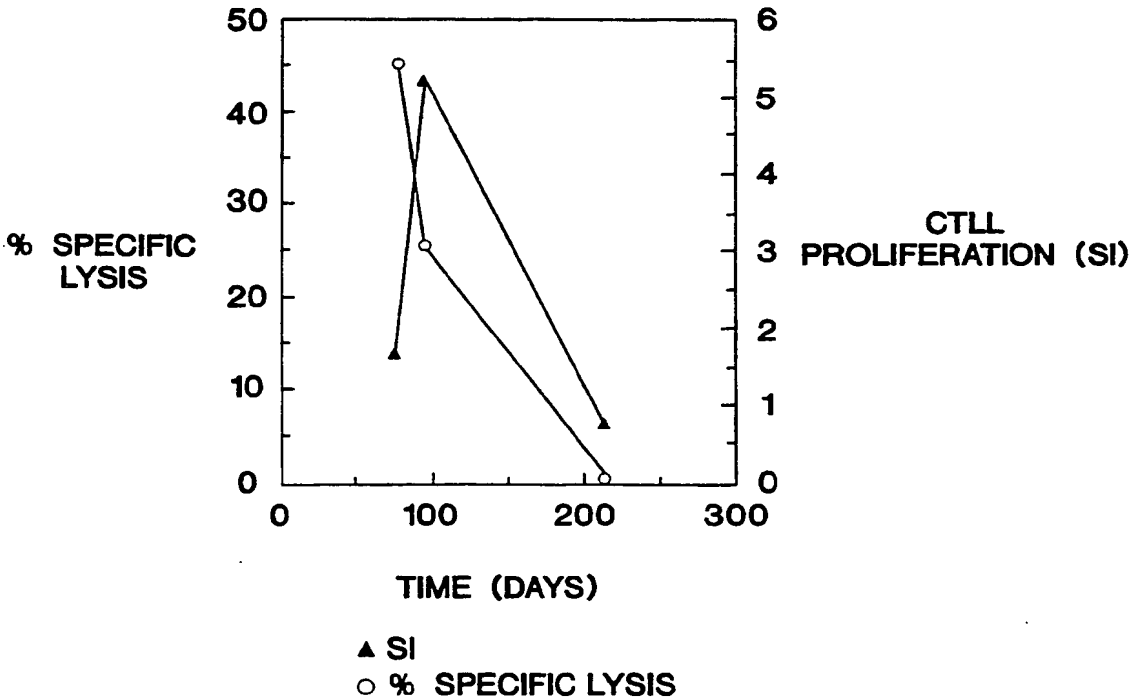


FIG. 6E

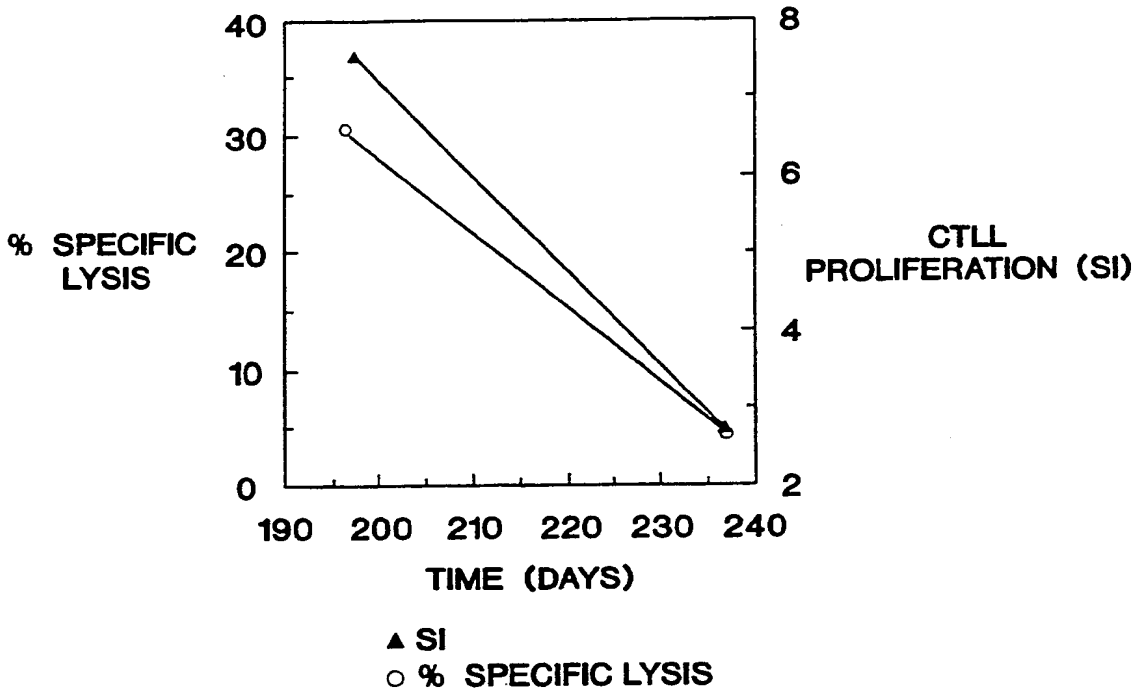


FIG. 7A

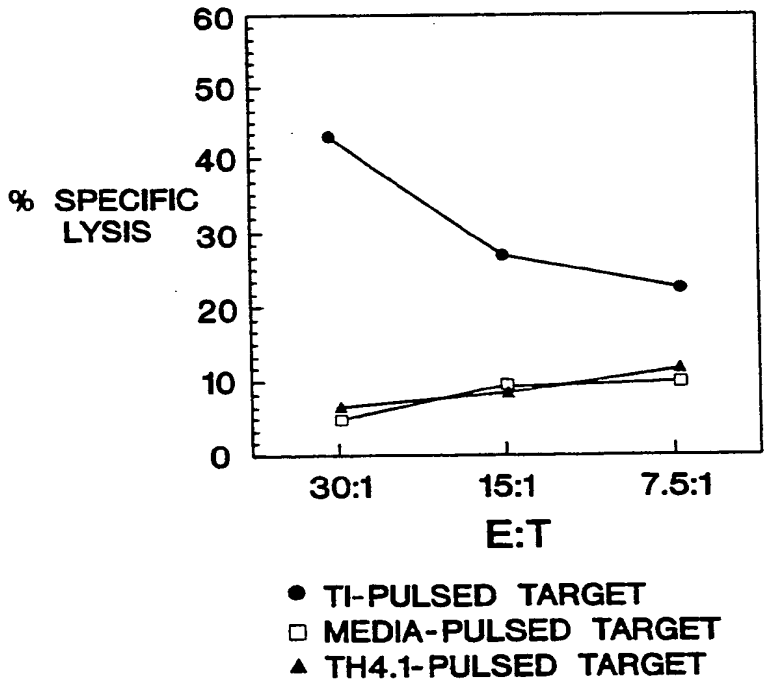


FIG. 7B

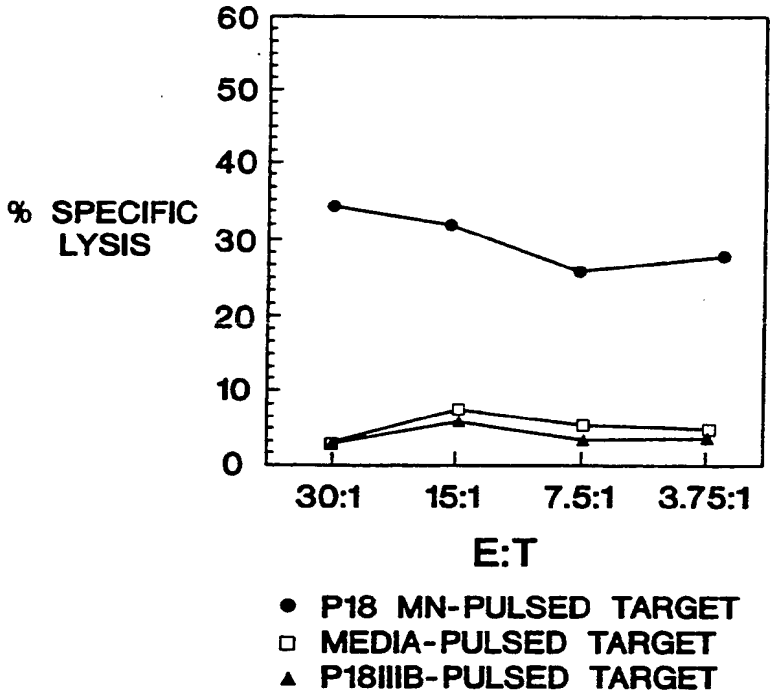
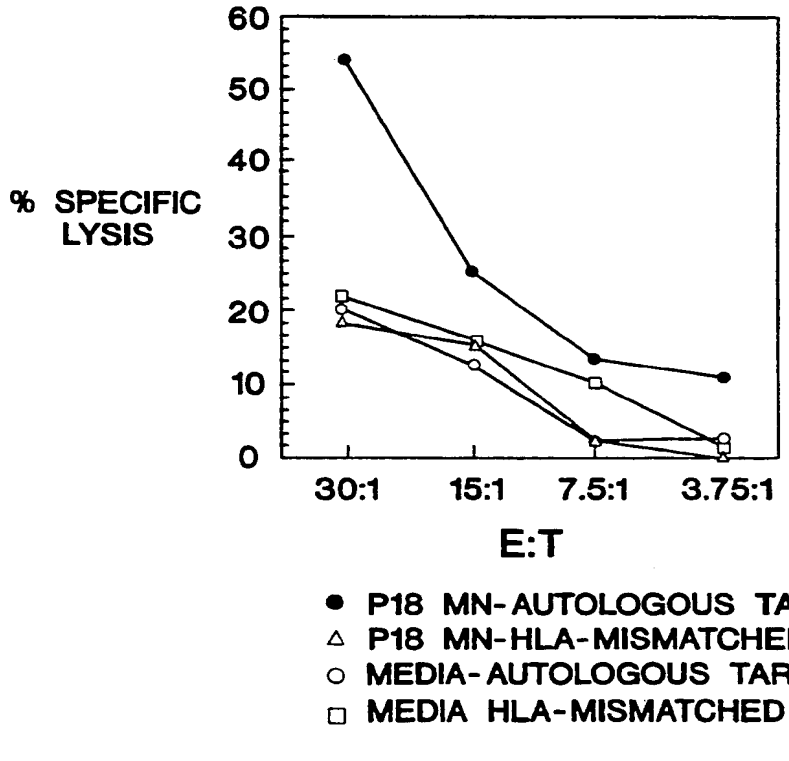


FIG. 7C



INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 96/10108

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/569 G01N33/68 C07K14/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION JAMA, vol. 271, no. 1, 5 January 1994, CHICAGO IL USA, pages 42-46, XP000605951 M. CLERICI ET AL.: "HIV-specific T-helper activity in seronegative health care workers exposed to contaminated blood" see the whole document --- -/--	1-14, 19-25

[X] Further documents are listed in the continuation of box C.

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22 October 1996	04. 11. 96

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	THE JOURNAL OF INFECTIOUS DISEASES, vol. 164, no. 1, 1 July 1991, CHICAGO IL USA, pages 178-182, XP000605948 M. CLERICI ET AL.: "Exposure to human immunodeficiency virus type 1. Specific T helper cell response before detection of infection by polymerase chain reaction" cited in the application see the whole document ---	15-18
Y	US,A,5 081 226 (J.A. BERZOFSKY ET AL.) 14 January 1992 cited in the application see claims 1-5 -----	15-18

1

INTERNATIONAL SEARCH REPORT

formation on patent family members

Intern·tional Application No
PCT, US 96/10108

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5081226	14-01-92	AU-B-	592258	04-01-90
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		EP-A-	0273716	06-07-88
		JP-B-	7062031	05-07-95
		JP-T-	63503227	24-11-88
		WO-A-	8805051	14-07-88

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A1

(11) International Publication Number: **WO 96/41189**

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(21) International Application Number: PCT/US96/10108

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(30) Priority Data:
08/488,435 7 June 1995 (07.06.95) US

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(72) Inventors; and
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(74) Agents: SVENSSON, Leonard, R. et al.; Birch, Stewart, Kolasch & Birch, L.L.P., P.O. Box 747, Falls Church, VA 22040-0747 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
With amended claims.

Date of publication of the amended claims:
6 February 1997 (06.02.97)

(54) Title: TEST OF HIV-SPECIFIC T LYMPHOCYTE FUNCTION THAT DETECTS EXPOSURE TO HIV ANTIGENS AND POSSIBLY EARLY HIV INFECTION

(57) Abstract

Methods for the detection of a T cell response in a patient to an antigen from an exogenous source are described. The antigen may be from any non-self source, but the method is particularly advantageous for detection of exposure to agents which do not produce rapid antibody responses. The method is particularly advantageous in detecting exposure to HIV and to other agents where early detection of exposure is important. The method detects activation of T cells in the absence of an antibody response.

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AMENDED CLAIMS

[received by the International Bureau on 06 January 1997 (06.01.97);
original claims 1, 2 and 19 amended and renumbered as claims 1-3;
original claims 5, 6, 15 and 17 renumbered as claims 4-7;
remaining claims cancelled (2 pages)]

1. A method for diagnosing exposure of a patient to an infectious agent which comprises:

a) obtaining peripheral blood mononuclear cells from a patient;

b) incubating said peripheral blood mononuclear cells with at least one synthetic peptide representing an epitope(s) of said infectious agent; and

c) measuring the killing activity of cytotoxic T lymphocytes of said peripheral blood mononuclear cells as a result of the incubation in step (b).

2. A method for diagnosing exposure of a patient to an infectious agent which comprises:

a) identifying at least one peptide epitope present in antigens of said infectious agent which provoke an immune response in a mammal;

b) obtaining peripheral blood mononuclear cells from a patient;

c) incubating said peripheral blood mononuclear cells with at least one synthetic peptide representing the epitope(s) of step (a); and

d) measuring the killing activity of cytotoxic T lymphocytes of said peripheral blood mononuclear cells as a result of the incubation in step (c).

3. A method for diagnosing exposure of a patient to an infectious agent which comprises:

a) identifying peptide epitopes present in antigens of said infectious agent which provoke an immune response in a mammal;

b) obtaining mononuclear cells from the lymph nodes or spleen of a patient;

c) incubating said mononuclear cells from step (2) with synthetic peptides representing the epitopes of step 1; and

d) measuring the killing activity of cytotoxic T lymphocytes of said mononuclear cells a result of the incubation in step (3).

4. The method of any one of claims 1 to 3, wherein said infectious agent is a virus.

5. The method of any one of claims 1 to 3, wherein said infectious agent is Human Immunodeficiency Virus.

6. The method of any one of claims 1 to 3 and 5, wherein the peptides are chosen from the group consisting of KQIINMWQEVGKAMYA, HEDIISLWDQSLK, DRVIEVVQGAYRAIR, RIQRGPGRAFVTIGK, and RIHIGPGRAFYTTKN.

7. The method of any one of claims 1 to 6, wherein a positive diagnosis is indicated by positive response to at least two peptides.

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